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(54) Title: VERTEBRATE SLIT DNA SEQUENCE, PROTEIN AND USES THEREOF

(57) Abstract

Polynucleotides that encode the Xenopus slit protein along with the deduced amino acid sequence of the Xenopus slit protein are given. Recombinant polynucleotides, vectors and transformed cells containing the slit polynucleotide are disclosed. Methods for production of slit proteins, pharmaceutical compounds containing slit protein and therapeutic uses for slit protein are also given. Methods for the repulsive guidance of nerve axon growth and inhibition of cell migration using slit are provided. Methods of stimulating cell proliferation using slit are given, along with methods and compositions for reducing the use of serum in cell culture by the use of slit.

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VERTEBRATE SLIT DNA SEQUENCE, PROTEIN AND USES THEREOF

CROSS–REFERENCE TO RELATED APPLICATIONS

This application claims priority from provisional application 60/124,767, filed March 17, 1999, which is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND

This invention relates to newly identified and isolated polynucleotides, proteins encoded by the polynucleotides, methods for producing the proteins and uses for the polynucleotides and proteins. More particularly the invention relates to the vertebrate slit proteins, polynucleotides encoding vertebrate slit proteins, methods for producing vertebrate slit proteins and the uses of vertebrate slit proteins and polynucleotides. More particularly still, the invention relates to Xenopus slit proteins and polynucleotides, along with their production and uses.

Cell migration is essential in species ranging from bacteria to humans (for recent reviews, see Mitchison and Cramer, *Cell*, 84:371-379 (1996); Lauffenburger and Horwitz, *Cell*, 84:359-369 (1996); Montell, *Development*, 126:3035-3046 (1999)). In the amoebae *Dictyostelium discoideum*, cell migration is involved in chemotaxis towards food sources and in cell aggregation and differentiation (reviewed in Devreotes and Zigmond, *Ann. Rev. Cell Biol.*, 4:469-686 (1988); Parent and Devreotes, *Ann. Rev. Biochem.*, 65:411-440 (1996); Chen et al., *Trends Genet.*, 12:52-57 (1996); Parent and Devreotes, *Science*, 284:765-770 (1999)). In higher vertebrates, cell migration plays important roles in multiple physiological and pathological processes. During embryonic and neonatal development, cell migration is crucial for morphogenetic movement such as gastrulation, cardiogenesis, formation of the internal organs such as the lung and kidney, formation of hematopoietic organs, and the formation of the central and peripheral nervous systems (CNS and PNS). In adult animals, cell migration is required for leukocyte trafficking and inflammatory responses. In tumor growth and development, tumor-induced angiogenesis and tumor metastasis both involve cell migration. Despite its importance, our understanding of mechanisms underlying cell migration in mammals is still limited (Lauffenburger and Horwitz, *Cell*, 84:359-369 (1996); Mitchison and Cramer, *Cell*, 84:371-379 (1996)).

Neural development is the process by which the cells of an embryo organize themselves into a complete and functioning nervous system. A striking feature of neural development is that the majority of neurons in the developing nervous system have to migrate to reach their final positions. Studies of neuronal migration are important for our understanding of the formation of the normal nervous system and for understanding the etiology of human diseases caused by abnormal migration and to provide a basis for designing therapeutic approaches to neurological diseases.

Neuronal migration in the developing central nervous system (CNS) was initially inferred from histological observations by classical neuroembryologists. The question of whether cells truly migrated or whether cells formed earlier were simply displaced by cells formed later was clarified by observations of changes of cell position in a direction opposite to that expected from cell displacement through histological examinations in the spinal cord of chick embryos and more convincingly by autoradiographic tracing in the cerebrum of rodent embryos. The fact that only nuclei were traced in autoradiographic studies raised the possibility that nuclei, but not entire cells, moved in the highly structured nervous system. Electron microscopic (EM) examination and reconstruction uncovered strong evidence for the migration of neuronal cell bodies. Observations of primary neurons and glia cultured *in vitro* demonstrated directly that neurons indeed migrate. Through a considerable amount of work with histological, autoradiographic, retroviral tracing, dye labeling and modern imaging techniques, it is now well established that the majority of neurons migrate throughout the developing nervous system.

In humans, proper migration of neurons is essential for the formation and normal functioning of the nervous system. Defects in neuronal migration can cause multiple clinically relevant diseases including epilepsy. Migration may also be important for invasion of tumors including neuroblastoma and glioblastoma and other tumors of the nervous system. Although it has been known for some time that cell migration is essential for postnatal behavior changes in birds, only recently have studies revealed that neurogenesis and neuronal migration continue in the brains of postnatal mammals, including humans. These findings also highlight that, for successful applications of cell-based therapies of neurodegenerative diseases, it is essential to direct correct migration of neurons or cells expressing therapeutic products to the target regions.

Our understanding of the molecular mechanisms guiding neuronal migration is still limited. Genetic studies in humans and in mice have led to the identification of multiple

molecules whose deficiencies cause defects in neuronal migration. Because most of these are intracellular molecules, it is unlikely that they can serve as guidance cues for migrating neurons. An interesting molecule identified from genetic studies is the secreted protein Reelin (D'Arcangelo et al., *Nature*, 374:719-723 (1995); Hirotsune et al., *Nat. Genet.*, 10:77-83 (1995)). Loss of function mutations in the *reeler* gene cause defects in central nervous system (CNS) lamination, and Reelin has been thought to control cell-cell interactions critical for cell positioning. It is not clear, however, whether Reelin acts as a stop signal or an adhesive molecule for migrating neurons. Because molecules can regulate neuronal migration indirectly, it is also not clear whether Reelin affects neuronal migration directly or indirectly. Similarly, the precise roles played by the other genetically identified molecules in neuronal migration remain to be understood.

An axon is a long projection of a nerve cell that generally carries nerve impulses away from the nerve cell body. Correct projection of axons to their targets is essential for the formation and function of the nervous system. Identification of axon guidance molecules and determination of their expression patterns and functional properties are therefore critical for understanding neural development. In addition, a thorough understanding of the molecular mechanisms involved in guiding developing axons to the proper location is crucial to the development of new technologies to regenerate damaged nerve tracts following traumatic injuries, such as spinal cord injuries, and in treatment of genetic conditions resulting in central nervous system malformation.

Previously, two families of secreted long-range chemoattractants and chemorepellents, the Netrins and Semaphorins (Sema) were known to function in directing axon projections (Tessier-Lavigne and Goodman, *Science* 274:1123-1133 (1996); Culotti and Kolodkin, *Curr. Opin. Neurobiol.* 6:81-88 (1996); Nieto, *Neuron*, 17:1039-1048 (1996); Puschel, *Eur. J. Neurosci.* 8:1317-1321 (1996); Varela-Echavarria and Guthrie, *Genes & Dev.* 11:545-557 (1997)).

Recently, a new family of proteins associated with the development of the nervous system, termed slit proteins, have been discovered. Slit mutations were originally observed in the fruit fly Drosophila in saturation mutagenesis experiments for mutations affecting larval cuticular patterning (Nusslein-Volhard et al., *Roux's Arch. Dev. Biol.*, 193:267-282 (1984)). Recently, vertebrate slit genes have been found in rats and humans (Nakayama et al., *Genomics* 51:27-34 (1998); Itoh et al., *Mol. Brain Res.* 62:175-186 (1998); Holmes et al.,

Mech. Devel. 79:57-72 (1998)). Drosophila slit cDNA was isolated by screening for genes encoding epidermal growth factor (EGF) repeats which hybridized to a probe made from Notch, a gene involved in cell fate determination (Rothberg et al., *Cell* 55:1047-1049 (1988)). Drosophila slit mRNA was found to be expressed in midline glial cells, whereas the secreted protein product of the slit mRNA was found in the midline cells and on axons traversing the midline cells (Rothberg et al., *Cell* 55:1047-1049 (1988); Rothberg et al., *Genes Dev.* 4:2169-2187 (1990)). Loss of slit function is thought to cause defects in the differentiation of midline cells and the separation of longitudinal axonal tracts (Rothberg et al., *Cell* 55:1047-1049 (1988); Rothberg et al., *Genes Dev.* 4:2169-2187 (1990)).

Molecular mechanisms underlying axon guidance at the midline appear to be conserved between vertebrates and invertebrates. The Netrin protein, which is secreted by the floor plate of the neural tube, is attractive to commissural axons in vertebrates, Drosophila and the roundworm C. elegans (Ishii et al., *Neuron* 9:873-881 (1992); Kennedy et al., *Cell* 78:425-435 (1994); Serafini et al., *Cell* 78:409-424 (1994); Harris et al., *Neuron* 17:217-228 (1996); Mitchell et al., *Neuron* 17:203-215 (1996); Kolodziej et al., *Cell* 87:197-204 1996)). Netrin can also act as a repellent for a specific subset of axons in C. elegans and vertebrates (Hedgecock et al., *Neuron* 4:61-84 (1990); Colamarino and Tessier-Lavigne, *Cell* 81:621-629 (1995)). Recent studies in Drosophila have revealed that a transmembrane receptor encoded by the *roundabout* (*robo*) gene plays an important role in ensuring that commissural axons which have already crossed the midline do not re-cross the midline and that other axons which stay ipsilateral do not cross the midline (Seeger et al., *Neuron* 10:409-426 (1993); Kidd et al., *Cell* 92:205-215 (1998); Kidd et al., *Nueron* 20:25-33 (1998); Zallen et al., *Cell* 92:217-227 (1998)). The phenotype of *robo* mutants and the predicted molecular features of the robo protein suggest the existence of a ligand for robo at the midline. Thus, although the robo protein was known and thought to function as a transmembrane receptor, the ligand for the robo protein remained unknown. The slit protein of the present invention is the first known ligand for the robo protein.

The olfactory system provides a useful model for studying neuronal migration and axon guidance. The olfactory bulb (OB) is a structure relaying olfactory information from the olfactory epithelium to the primary olfactory cortex. The major types of interneurons in the OB, including the granule cells and the periglomerular cells, are produced postnatally from the anterior part of the subventricular zone (SVZa) of the telencephalon in rodents.

Neuronal progenitors thus have to migrate in the rostral migratory stream (RMS) from the SVZa to the OB.

The importance of cell migration in normal development and homeostasis extends beyond the nervous system. One area in which cell migration plays an important role is in the immune system and especially in the area of cell-mediated immunity. The term cell-mediated immunity originally described localized immune reactions to organisms mediated by leukocytes. The term, however, has also been used in a more general sense for any immune response in which antibodies play a subordinate role.

Leukocyte chemotaxis was first described by Leber in 1888 (reviewed in McCutcheon, *Physiol. Rev.*, 26:319-336 (1946); Harris, *Physiol. Rev.*, 34:529-562 (1954)) and is one of the best-studied phenomena of cell migration in adult mammals (Boyden, *J. Exp. Med.*, 115:453-466 (1962); Ramsey, *Exp. Cell Res.*, 70:129-139 (1972); Zigmond, *Nature*, 249:450-452 (1974); Devreotes and Zigmond, *Ann. Rev. Cell Biol.*, 4:649-686 (1988); Downey, *Immunol.*, 6:113-124 (1994); Sanchez-Madrid and Angel del Pozo, *EMBO J.*, 18:501-511 (1999)). Work in the past decade has shown that the chemokines family are attractive guidance cues for leukocytes (reviewed in Murphy, *Ann. Rev. Immunol*, 12:593-633 (1994); Springer, *Cell*, 76:301-314 (1994); Rollins, *Blood*, 90:909-928 (1997); Baggiolini et al., *Ann. Rev. Immunol.*, 15:675-705 (1997); Luster, *N. Eng. J. Med.*, 338:436-445 (1998); Locati and Murphy, *Ann. Rev. Med.*, 50:425-440 (1999)). The first chemokine was isolated and sequenced in 1977 without knowledge of its biological activity (Duel et al., *Proc. Natl. Acad. Sci. USA*, 24:2256-2258 (1977), and functional studies of the chemokines began in 1987 with the identification of IL-8 (Yoshimura et al., *Proc. Natl. Acad. Sci.*, 84:9233-9237 (1987). There are more than 40 chemokines known now and they are structurally related small proteins with 70 to 100 amino acid residues (Murphy, *Ann. Rev. Immunol*, 12:593-633 (1994); Rollins, *Blood*, 90:909-928 (1997); Baggiolini et al., *Ann. Rev. Immunol.*, 15:675-705 (1997); Luster, *N. Eng. J. Med.*, 338:436-445 (1998); Locati and Murphy, *Ann. Rev. Med.*, 50:425-440 (1999)). Although chemokines have been implicated in a number of other biological processes, their best-characterized roles are those in leukocyte chemotaxis. There are four families of chemokines: the CXC (or α) family with the first two N terminal cysteines separated by one non-conserved amino acid residue, the CC (or β) family with the first two cysteines unseparated, the C (or γ) family with one N terminal cysteine, and the CX_3C (or δ) family with the N terminal cysteines separated by three

residues. Among the best characterized chemokines are CC chemokine RANTES and CXC chemokine, stromal cell derived factor 1 (SDF-1) (Tashiro et al., *Science* 261:600-603 (1993)). Receptors for RANTES have been detected on monocyte, eosinophil, basophil, T lymphocytes, natural killer cells and dendritic cells. Receptors for SDF-1 have been found on monocyte, T lymphocytes and dendritic cells. Both chemokines are implicated in a number of inflammatory diseases.

During inflammation, it is well known that leukocyte chemotaxis induced by chemokines is essential for extravasation and trafficking to target tissues (e.g., Mohle et al., *Blood*, 91:4523-4530 (1998); Campbell et al., *Nature*, 400:776-780 (1999)). Allergic inflammation including those in the airways such as asthma affects a large number of people (Holtzman et al., "Asthma" in *Principles of Molecular Medicine*, Jameson, ed., Humana Press, 1998, pp. 319-327). Migration to, and accumulation of, leukocytes in inflammatory sites are crucially involved in allergic inflammation (reviewed in Seminario and Gleich, *Curr. Opin. Immunol.*, 6a;860-864 (1994); Baggiolini and Dahinder, *Immunology Today*, 15:127-133 (1994); Holtzman et al., "Asthma" in *Principles of Molecular Medicine*, Jameson, ed., Humana Press, 1998, pp. 319-327)). Expression of chemokines is up-regulated in allergic inflammation including asthma (Sousa et al., *Am. J. Respir. Cell. Mol. Biol.* 10:142-147 (1994); MacLean et al., *J. Exp. Med.* 184:1461-1469 (1996); Ganzalo et al., *Immunity*, 4:1-14 (1996); Teran et al., *J. Immunol.*, 157:1806-1812 (1996)). Chemokines can attract leukocytes and activate them, causing mast cell degranulation and histamine release (Dahinder et al., *J. Exp. Med.*, 179:751-756 (1994); Luster and Rothenberg, *J. Leukoc. Biol.*, 62:620-633 (1997)). Inhibition of chemokine signaling by function-blocking antibodies or gene knock-out is effective against airway inflammation including asthma (Harada et al., *J. Leukoc. Biol.*, 56:559-564 (1994); Teran et al., *J. Immunol.*, 157:1806-1812 (1996); Lukacs et al., *J. Immunol.*, 158:4398-4404 (1997); Rothenberg et al., *J. Exp. Med.*, 185:785-790 (1997)). Increased production of chemokines has been implicated in arthritis (for example, Koch et al., *J. Clin. Invest.*, 90:772-779 (1992)). Inhibition of the chemokines IL-8, MCP-1 or RANTES has been demonstrated to ameliorate arthritis in rabbit (Harada et al., *J. Leukoc. Biol.*, 56:559-564 (1994)), mouse (Gong et al., *J. Exp. Med.*, 186:131-137 (1997)) and rat models (Barnes et al., *J. Clin. Invest.*, 101:2910-2919 (1998)). Chemokines are also implicated in glomerulonephritis (Harada et al., *J. Leukoc. Biol.*, 56:559-564 (1994); Brown et al., *J. Leukoc. Biol.*, 59:75-80 (1996)), inflammatory responses to viruses (Cook et al., *Science*,

269:1583-1585 (1995)), PNS demyelination (Fujika et al., *J. Neurovirol.*, 5:27-31 (1999); Glabinski and Ransohoff, *J. Neurovirol.*, 5:3-12 (1999)), and chronic inflammatory responses such as those in cystic fibrosis (reviewed in Rosenbluth and Brody, "Cystic Fibrosis" in *Principles of Molecular Medicine*, Jameson, ed., Humana Press, 1998, pp. 329-338), or in Ulcerative colitis and Crohn's diseases (Reinecker, et al., *Gastroenterology*, 108:40-50 (1995); Grimm et al., *J. Gastroenterol. Hepatol.*, 10:387-395 (1995); Garcia-Zepeda et al., *Nat. Med.*, 2:449-456 (1996)).

In the nervous system, chemokines have been implicated in a number of diseases including encephalitis, encephalomyelitis, meningitis, CNS ischemia, CNS reperfusion injury, CNS trauma, CNS tumor, multiple sclerosis, Alzheimer's diseases and HIV dementia (reviewed in Karpus, *J. Neurovirol.*, 5:1-2 (1999); Glabinski and Ransohoff, *J. Neurovirol.*, 5:3-12 (1999); Fujika et al., *J. Neurovirol.*, 5:27-31 (1999); Hesselgesser and Horuk, *J. Neurovirol.*, 5:13-26 (1999); Xia and Hyman, *J. Neurovirol.*, 5:32-41 (1999); Mennicken et al., *Trends Pharmacol Sci.*, 20:73-78 (1999)). Multiple sclerosis is an autoimmune disease mediated by leukocyte infiltration. Chemokine expression is up-regulated in multiple sclerosis or its animal models (Ransohoff et al., *FASEB J.*, 7:592-600 (1993); Miyagishi et al., *J. Neurol. Sci.*, 129:223-227 (1995); Godiska et al., *J. Neuroimmunol.*, 58:167-176 (1995); Ransohoff et al., *Cytokine Growth Factor Rev.* 7:35-46 (1996); Ransohoff, *J. Leukoc. Biol.*, 62:645-652 (1997)); Hvas et al., *Scand. J. Immunol.*, 46:195-203 (1997); Glabinski and Ransohoff, *J. Neurovirol.*, 5:3-12 (1999); Hesselgesser and Horuk, *J. Neurovirol.*, 5:13-26 (1999)), and inhibition of chemokine receptors is protective against the induction of experimental allergic encephalomyelitis, a mouse model of multiple sclerosis (Karpus et al., *J. Immunol.*, 155:5003-5010 (1995)).

Chemokine receptors are essential for the infection of intracellular pathogens including HIV-1 (Cocchi et al., *Science*, 270: 1811-5 (1995); Feng et al., *Science*, 272: 872-7 (1996); Oberlin et al., *Nature*, 382: 833-5 (1996); Bleul et al., *Proc. Natl. Acad. Sci*, 94: 1925-30 (1996, 1997); Dragic et al., *Nature*, 381: 667-73 (1996); Deng et al., *Nature*, 381: 661-6 (1996); Choe et al., *Cell*, 85: 1135-48 (1996); Doranz et al., *Cell*, 85: 1149-58 (1996); Arenzana-Seisdedos et al., *Nature*, 383: 400 (1996); Schmidtmayerova et al, *Nature*, 382: 767 (1996); Baggiolini, Dewald and Moser, *Annu. Rev. Immunol.*, 15: 675-705 (1997); Hori et al., *J. Immunol.*, 160: 180-8 (1998); Luster, *N. Engl. J. Med.*, 338: 436-45(1998); Locati and Murphy, *Annu. Rev. Med.* 50: 425-40 (1999); Horuk, *Immunol. Today*, 20: 89-94 (1999);

Berger et al., *Annu. Rev. Immunol.*, 17: 657-700 (1999)) and the malaria-causing protozoan *Plasmodium vivax* (Horuk et al., *Science*, 261: 1182-4 (1993)). Several chemokine receptors including CXCR4 and CCR5 have been shown to be co-receptors for HIV (Cocchi et al., *Science*, 270: 1811-5 (1995); Feng et al., *Science*, 272: 872-7 (1996); Oberlin et al., *Nature*, 382: 833-5 (1996); Bleul et al., *Proc. Natl. Acad. Sci*, 94: 1925-30 (1996, 1997); Dragic et al., *Nature*, 381: 667-73 (1996); Deng et al., *Nature*, 381: 661-6 (1996); Choe et al., *Cell*, 85: 1135-48 (1996); Doranz et al., *Cell*, 85: 1149-58 (1996); Simmons et al., *Science*, 276: 276-9 (1997)) and inhibition of receptor signaling has been shown to block HIV infection (Alfano et al., *J. Exp. Med.*, 190: 597-605 (1999); Wang and Oppenheim, *J. Exp. Med.*, 190: 591-5 (1999)).

Although it is clear that cell migration plays an important role in both the nervous and immune systems, it is much less clear if common mechanisms controlling and/or guiding cell migration exist between cell types. Molecularly, all known neuronal guidance cues function through single transmembrane receptors including UNC-5, DCC, Eph, neuropilin, Robo and plexin (Leung-Hagesteijn et al., *Cell*, 71: 289-299 (1992); Cheng and Flanagan, *Cell*, 79: 157-168 (1994); Keino-Masu et al., *Cell*, 87: 175-185 (1996); Chan et al., *Cell*, 87: 187-195 (1996); Leonardo et al., *Nature*, 386: 833-838 (1997); Ackerman et al., *Nature*, 386: 838-842 (1997); He and Tessier-Lavigne, *Cell* 90: 739-751 (1997); Kolodkin et al., *Cell,* 90: 753-762 (1997); Feiner et al., *Neuron* 19: 539-545 (1997); Chen et al., *Neuron* 21: 1283-1290 (1998); Giger et al., *Neuron*, 21: 1079-1092 (1998); Winberg et al., *Cell,* 95: 903-916 (1998); Kidd et al., *Cell,* 92:205-215 (1998); Kidd, et al., *Cell,* 96:785-794., (1999); Brose et al., *Cell,* 96:795-806 (1999); Li et al., *Cell* 96: 807-818 (1999); Yuan et al., *Dev. Biol.,* 212:290-306 (1999); Takahashi et al., *Cell* 99:59–69 (1999); Tamagnone et al., *Cell* 99:71–80 (1999); Bashaw and Goodman, *Cell*, 97:917–926 (1999)). In contrast, seven transmembrane receptors coupled to G proteins (GPCR) are required for all chemokines and other chemotactic factors for leukocytes (Hwang, *J. Lipid Mediat.* 2:123-58 (1990); Murphy, *Ann. Rev. Immunol.*, 12:593-633 (1994); Rollin, *Blood*, 90:909-928 (1997); Baggiolini et al., *Annu. Rev. Immunol.* 15:675-705 (1997); Luster, *N. Engl. J. Med.,* 338:436-45 (1998); Locati and Murphy, *Annu. Rev. Med.,* 50:425-40 (1999)) and *Dictyostelium* (Devreotes and Zigmond, *Ann. Rev. Cell Biol.* 4:649-686 (1988); Parent and Devreotes, *Ann. Rev. Biochem.*, 65:411-440 (1996); Chen et al., *Trends Genet.*, 12:52-57 (1996); Parent and Devreotes, *Science*

84:765-70 (1999)). Thus, there is no clear connection between the mechanisms controlling migration in the nervous and immune systems.

SUMMARY

Accordingly, the present invention provides a polynucleotide encoding the sequence for the vertebrate form of the slit protein as well as recombinant polynucleotides containing the nucleic acid sequence for the slit protein, recombinant vectors and expression cassettes containing the nucleic acid sequence for the slit protein. Also provided are the protein encoded by the polynucleotides of the present invention, host cells that have been genetically transformed with the polynucleotides of the present invention, and methods for producing the slit protein. Additionally the invention provides for pharmacological compounds containing the slit protein whose uses include, but are not limited to, the guidance of axon projection and neuronal migration, leukocyte migration, and the study of central nervous system development. Also, the present invention provides for the therapeutic use of slit proteins in directing axon and dendrite growth and projections during nerve regeneration, in inhibiting the migration of malignant cells, in modulating the migration of leukocytes to inhibit inflammation and graft rejection, and in inhibiting of infection of cell by HIV. Additionally the invention provides for the use of slit for increasing cell proliferation, the use of slit as a serum replacement in cell culture medium and cell culture media containing slit proteins. This invention also provides for the use of slit in wound-healing and organ repair such as regeneration or repair of lung and kidney.

Accordingly one aspect of the invention is to provide an isolated polynucleotide comprising a member selected from the group consisting of (a) a polynucleotide of SEQ ID NO: 1, fragments of SEQ ID NO: 1, or the complements thereof; (b)a polynucleotide that has at least 90% sequence identity with the polynucleotide of (a); (c) a polynucleotide that hybridizes to the polynucleotide of (a) under conditions of 5X SSC, 50% formamide and 42°C, and which encodes a protein having the same biological function; (d) a polynucleotide encoding the same amino acid sequence as (a), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; (e) a polynucleotide encoding the same amino acid sequence as (b), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; and (f) a polynucleotide encoding the same amino acid

sequence as (c), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code.

Another aspect of the present invention is to provide a recombinant polynucleotide comprising a member selected from the group consisting of (a) a polynucleotide of SEQ ID NO: 1, fragments of SEQ ID NO: 1, or the complements thereof; (b)a polynucleotide that has at least 90% sequence identity with the polynucleotide of (a); (c) a polynucleotide that hybridizes to the polynucleotide of (a) under conditions of 5X SSC, 50% formamide and 42°C, and which encodes a protein having the same biological function; (d) a polynucleotide encoding the same amino acid sequence as (a), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; (e) a polynucleotide encoding the same amino acid sequence as (b), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; and (f) a polynucleotide encoding the same amino acid sequence as (c), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code.

In another aspect of the present invention is provided a recombinant vector comprising a member selected from the group consisting of (a) a polynucleotide of SEQ ID NO: 1, fragments of SEQ ID NO: 1, or the complement thereof; (b)a polynucleotide that has at least 90% sequence identity with the polynucleotide of (a); (c) a polynucleotide that hybridizes to the polynucleotide of (a) under conditions of 5X SSC, 50% formamide and 42°C, and which encodes a protein having the same biological function; (d) a polynucleotide encoding the same amino acid sequence as (a), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; (e) a polynucleotide encoding the same amino acid sequence as (b), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; and (f) a polynucleotide encoding the same amino acid sequence as (c), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code.

A further aspect of the invention is to provide host cells comprising the recombinant vector described above.

Yet another aspect is a protein or polypeptide encoded by a polynucleotide selected from the group comprising a member selected from the group consisting of (a) a polynucleotide of SEQ ID NO: 1; fragments of SEQ ID NO: 1, or the complements thereof; (b)a polynucleotide that has at least 90% sequence identity with the polynucleotide of (a); (c)

a polynucleotide that hybridizes to the polynucleotide of (a) under conditions of 5X SSC, 50% formamide and 42°C, and which encodes a protein having the same biological function; (d) a polynucleotide encoding the same amino acid sequence as (a), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; (e) a polynucleotide encoding the same amino acid sequence as (b), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; and (f) a polynucleotide encoding the same amino acid sequence as (c), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code.

Still another aspect of the invention is a protein comprising the amino acid sequence of SEQ ID NO: 2 or a fragment of said protein.

Another aspect provides a method for the production of isolated slit protein comprising growing transformed host cells of the present invention under conditions where the cells express slit protein and then isolating the expressed protein or its fragments.

In a further aspect is provided, a pharmaceutical composition comprising the protein or protein fragment of the present invention or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier, diluent, or excipient.

In an additional aspect is provided a method for altering cell migration comprising administering a migration-altering amount of a slit protein or a pharmaceutically acceptable salt thereof.

Yet another aspect provides a method for guiding cell migration comprising administering a cell migration guiding amount of slit protein or a pharmaceutically acceptable salt thereof.

Yet a further aspect provides a method for guiding nerve axons or dendrites comprising administering a axon- or dendrite-guiding amount of a slit protein or a slit protein derivative.

Another aspect provides a method for treating graft rejection comprising administering a leukocyte migration inhibiting amount of a slit protein or a slit protein derivative.

A further aspect provides a method for increasing cell proliferation by transforming the cells with a vector containing the polynucleotide of the present invention.

Additional aspects include a method for culturing cells comprising the addition of a slit protein or a slit protein derivative to the culture medium as well as a culture medium comprising a slit protein or a slit protein derivative.

Still another aspect is a composition comprising the slit protein of the present invention.

Also provided is an expression cassette comprising the polynucleotide of the present invention.

Additionally is provided a method for inhibiting the infection of cells by the HIV virus comprising administering an infection inhibiting amount of slit protein or a slit protein derivative.

Further provided is a method for aiding wound repair, organ repair or organ regeneration comprising administering a cell proliferating amount of a slit protein or a slit protein derivative.

In addition, is provided a method for treating inflammation comprising administering a leukocyte migration inhibiting amount of a slit protein.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims and accompanying figures where:

Figure 1 shows nucleotide sequence of the slit protein of the present invention (SEQ ID NO: 1).

Figure 2 shows the amino acid sequence deduced from the nucleotide sequence of the present invention (SEQ ID NO: 2).

Figure 3 shows the cDNA sequence of human slit-2 (SEQ ID NO:3) as contained in GenBank (accession number AF055585).

Figure 4 shows the repulsive effect of Slit on neurons migrating from SVZa explants. **a-c**. Distribution of chains of cells from the SVZa explants, co-cultured with an aggregate of control HEK cells (a), with cells expressing xSlit (b) or mSlit-1 (c) in the matrigel for one (a, b) or two days (c). d-f. Distribution of cells migrating out of the SVZa explants after being co-cultured with control HEK cells (d), with cells expressing xSlit (e) or mSlit-1 (f) in the collagen gel.

Figure 5 shows the effect of slit on neuronal migration in the RMS. DiI crystals were placed into SVZa in sagittal sections of postnatal rat brains to label cells (red in b, c, e and f) migrating into the RMS. Control or Slit HEK cells were labeled with DiO (green in a, c, d and f). a-c are different views of the same slice in which an aggregate of control HEK cells were placed on top of the RMS. d-f are different views of the same slice in which an aggregate of Slit cells were placed on top of the RMS. In all panels, the upper right corner is towards the SVZa, the origin of the RMS, whereas the lower left corner is towards to the OB, the end of the RMS.

Figure 6 shows the effect of Slit on Cells Migrating from LGE Explants. (A) Symmetric distribution of cells around the LGE explant cocultured with an aggregate of cells transfected with the vector (n= 87). (B) Symmetric distribution of migrating LGE cells when cocultured with an aggregate of human Sema III–expressing cells (n= 25). (C) Asymmetric distribution of migrating LGE cells when cocultured with an aggregate of mSlit1-expressing cells (n = 74). (D) Asymmetric distribution of migrating LGE cells when cocultured with an aggregate of xSlit-expressing cells (n = 89). (E) Symmetric migration of endothelial cells from an aorta explant in the presence of control HEK cells. (F) Symmetric migration of endothelial cells from an aorta explant in the presence of HEK cells expressing xSlit. (G). A diagram of regions in the rat telencephalon, showing the subventricular zone in the LGE from which explants were isolated and cultured, the ventricular zone, and the mantle layer of LGE.

Figure 7 shows the effect of slit on migration of cells from the LGE to the Neocortex. Results from E16.5 slices are shown here. Similar results have been obtained with E15.5 and E17.5 slices. (A–D) Different views of the same explant on which a strip of Slit cells (green) has been placed on the junction between the neocortex and the LGE. (E–H) Different views of the same explant on which a strip of control cells (green) has been placed on the junction between the neocortex and the LGE. (A) and (E) are Hoechst dye staining to show outlines of the brain slices. (B) and (F) show DiO-labeled aggregates of Slit (B) or control (F) cells; the green bands are the aggregates. (C) and (G) show DiI-labeled LGE neurons; note that, in the neocortex, there were migrating cells in (G) but not in (C). (D) is the superimposition of (A), (B), and (C), whereas (H) is the superimposition of (E), (F), and (G); note the relationship of HEK cells (green), the LGE (red) and migrating neurons in the neocortex (red), and the outlines of the slices (blue).

Figure 8 shows the inhibition of RANTES-induced lymph node cell migration by slit protein.

Figure 9 shows the inhibition of SDF-1-induced lymph node cell migration by slit protein.

Figure 10 shows cell migration in control or robo-overexpressing HEK cells that were transfected with CCR5.

Figure 11 shows cell migration in control or robo-overexpressing HEK cells that were transfected with CXCR4.

Figure 12 shows the effect of recombinant human slit 2 protein on inhibiting RANTES-induced lymph-node migration.

Figure 13 shows the effect of recombinant human slit-2 protein on inhibition of SDF-1 induced lymph-node cell migration

ABBREVIATIONS AND DEFINITIONS

AP = alkaline phosphatase

HA = hemagglutinin

PEG = polyethylene glycol

FCS = fetal calf serum

HEK = human embryonic kidney

HIV = human immunodeficiency virus

SVZa = subventricular zone

RMS = rostral migratory stream

OB = olfactory bulb

LGE = lateral ganglionic eminence

RANTES = regulated on activation, normal T cell expressed and secreted protein

SDF-1 = stromal derived factor 1

DMEM = Dulbecco's modified Eagle's medium

PBS = phosphate buffered saline

PCR = polymerase chain reaction

As used herein in reference to the slit protein of the present invention, "biological function" means the ability of a slit protein to guide cell migration as in Example 10; or the ability to repulse axon projection as in Example 8; or the ability of a slit protein to

specifically bind to the robo protein as characterized in Example 7; or by the ability of a slit protein to inhibit cytokine induced migration of leukocytes as in Example 11 or the ability to affect cell proliferation as in Example 12.

As used herein, the terms "complementary" or "complementarity" refer to the pairing of bases, purines and pyrimidines, that associate through hydrogen bonding in double stranded nucleic acid. The following base pairs are complementary: guanine and cytosine; adenine and thymine; and adenine and uracil. The terms as used herein the terms include complete and partial complementarity.

As used herein, the term "hybridization" refers to a process in which a strand of nucleic acid joins with a complementary strand through base pairing. The conditions employed in the hybridization of two non-identical, but very similar, complementary nucleic acids varies with the degree of complementarity of the two strands and the length of the strands. Thus the term contemplates partial as well as complete hybridization. Such techniques and conditions are well known to practitioners in this field.

As used herein, the term "amino acid" is used in its broadest sense, and includes naturally occurring amino acids as well as non-naturally occurring amino acids, including amino acid analogs and derivatives. The latter includes molecules containing an amino acid moiety. One skilled in the art will recognize, in view of this broad definition, that reference herein to an amino acid includes, for example, naturally occurring proteogenic L-amino acids; D-amino acids; chemically modified amino acids such as amino acid analogs and derivatives; naturally occurring non-proteogenic amino acids such as norleucine, β-alanine, ornithine, etc.; and chemically synthesized compounds having properties known in the art to be characteristic of amino acids.

As used herein, the term "proteogenic" indicates that the amino acid can be incorporated into a peptide, polypeptide, or protein in a cell through a metabolic pathway.

As used herein, the term "pharmaceutically acceptable salts" embraces salts commonly used to form alkali metal salts and addition salts of free acids or free bases. The nature of the salt is not critical, provided that it is pharmaceutically acceptable.

As used herein, "expression cassette" means a genetic module comprising a gene and the regulatory regions necessary for its expression, which may be incorporated into a vector.

As used herein, "secretion sequence" or "signal peptide" or signal sequence" means a sequence that directs newly synthesized secretory or membrane proteins to and through

membranes of the endoplasmic reticulum, or from the cytoplasm to the periplasm across the inner membrane of bacteria, or from the matrix of mitochondria into the inner space, or from the stroma of chloroplasts into the thylakoid. Fusion of such a sequence to a gene that is to be expressed in a heterologous host ensures secretion of the recombinant protein from the host cell.

As used herein, a "recombinant nucleic acid" is defined either by its method of production or its structure. In reference to its method of production, e.g., a product made by a process, the process is use of recombinant nucleic acid techniques, e.g., involving human intervention in the nucleotide sequence, typically selection or production. Alternatively, it can be a nucleic acid made by generating a sequence comprising fusion of two fragments which are not naturally contiguous to each other, but is meant to exclude products of nature, e.g., naturally occurring mutants. Thus, for example, products made by transforming cells with any unnaturally occurring vector is encompassed, as are nucleic acids comprising sequences derived using any synthetic oligonucleotide process. Such is often done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a single genetic entity comprising a desired combination of functions not found in the commonly available natural forms. Restriction enzyme recognition sites are often the target of such artificial manipulations, but other site specific targets, e.g., promoters, DNA replication sites, regulation sequences, control sequences, or other useful features may be incorporated by design.

As used herein, "polynucleotide" and "oligonucleotide" are used interchangeably and mean a polymer of at least 2 nucleotides joined together by phosphodiester bonds and may consist of either ribonucleotides or deoxyribonucleotides.

As used herein, "sequence" means the linear order in which monomers occur in a polymer, for example, the order of amino acids in a polypeptide or the order of nucleotides in a polynucleotide.

As used herein, "peptide" and "protein" are used interchangeably and mean a compound that consists of two or more amino acids that are linked by means of peptide bonds.

As used herein "recombinant protein" means that the protein, whether comprising a native or mutant primary amino acid sequence, is obtained by expression of a gene carried by a recombinant DNA molecule in a cell other than the cell in which that gene and/or protein is naturally found. In other words, the gene is heterologous to the host in which it is expressed. It should be noted that any alteration of a gene, including the addition of a polynucleotide encoding an affinity purification moiety to the gene, makes that gene unnatural for the purposes of this definition, and thus that gene cannot be "naturally' found in any cell.

As used herein, "cytokines" means a group of substances from by an animal in response to infection. They are similar to hormones in their function in that they are produced in one cell and stimulate a response in another cell or stimulate in response in the cell in which they are produced in an autocrine fashion.

As used herein, the term "chemokines" refers to a large family of small structurally related 8- to 10- kDa cytokines that have two or more conserved cysteines forming disulfied bonds.

As used herein, the term "animal" includes human beings.

As used herein, "targeting sequence" means in the context of gene or polynucleotide insertion, a sequence which results in the gene or polynucleotide being inserted at a particular location by homologous recombination. In the context of proteins or peptides, "targeting sequence" refers to a nucleotide sequence encoding an amino acid sequence the presence of which results in a protein being directed to a particular destination within a cell.

DETAILED DESCRIPTION

The following detailed description is provided to aid those skilled in the art in practicing the present invention. Even so, this detailed description should not be construed to unduly limit the present invention as modifications and variation in the embodiments discussed herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

All publications, patents, patent applications and other references cited in this application are herein incorporated by reference in their entirety as if each individual publication, patent, patent application or other reference were specifically and individually indicated to be incorporated by reference.

A polynucleotide encoding the slit protein of the frog genus Xenopus has been isolated from a Xenopus embryonic cDNA library. The sequence of the polynucleotide has been determined and is given in SEQ ID NO: 1. The cDNA sequence consists of 5513 bases and exhibits 59% sequence identity with the murine slit-2 sequence. Although a particular embodiment of the nucleotide sequence disclosed herein is given in SEQ ID NO: 1, it should be understood that other biologically functional equivalent forms of the nucleic acid sequence of the present invention can be readily isolated using conventional DNA-DNA and DNA-RNA hybridization techniques. Thus the present invention also includes nucleotide sequences that hybridize to SEQ ID NO: 1 or its complement under moderate to high stringency conditions and encode proteins exhibiting the same or similar biological activity as that of protein of SEQ ID NO: 2 disclosed herein. Also included in the invention are polynucleotides that exhibit 90%, preferrably 92%, more preferrably 95% ,and more 98% sequence identity with SEQ ID NO: 1, its complement or SEQ ID NO: 2. Such nucleotide sequences preferably hybridize to the nucleic acid of SEQ ID NO: 1 or its complement under high stringency conditions. Exemplary conditions include initial hybridzation in 5X SSPE,1-5X Denhardt's solution, 10-200 μg/ml denatured heterologous DNA, 0.5% SDS, at 50- 68°C for a time sufficient to permit hybridization, e.g. several hours to overnight, followed two washes in 2X SSPE, 0.1% SDS at room temperature and two additional 15 minute washes in 0.1X SSPE, 0.1% SDS at 42°C followed by detection of the hybridization products. Higher stringency washing can accomplished by at least one additional wash in 0.1% SSPE, 0.1% SDS at 55°C, more preferably at 60°C and more preferably still at 65°C. High stringency hybridizations can also be carried out in 5X SSPE and 50% formamide at 42°C followed by washing as described above (Meinkoth and Wahl, *Anal. Biochem*, 138:267-284 (1984)). As is well known by those of ordinary skill in the art, SSC can be substituted for SSPE in the above examples so that, for instance, hybridization can be accomplished in 5X SSC in place of 5X SSPE.

It is well known to those of ordinary skill in the art that different compositions can result in equal stringency conditions for hybridization depending on well known factors such as the concentration of Na^+, the % formamide, the temperature, the T_m of the hybrid to be formed, and the composition of the hybrid, e.g. DNA-DNA, DNA-RNA, or RNA-RNA. Thus the invention also encompasses nucleotide sequence that hybridize under conditions equivalent to those described above.

The amino acid sequence of the protein encoded by the isolated polynucleotide sequence has been deduced and is given in Figure 2. The protein exhibits 87% amino acid identity with the mouse slit-2 protein 85% identity with the human slit-2 protein, 63% identity with the mouse slit-1 and mouse slit-3 proteins, 83% identity with a partial chicken slit protein, 67% with the rat slit protein rMEGF5, 63% with the rat slit protein rMEGF4 (Nakayama et al., *Genomics* 51:27-34 (1998)), and 40% identity with the Drosophila slit protein. At the amino (N) terminus, there is a putative signal peptide characteristic of secreted proteins. There are four leucine rich repeats (LRR), each surrounded by an N terminal and a carboxyl (C) terminal flanking region. The leucine rich repeats are followed by nine epidermal growth factor (EGF) repeats. Near the C terminus, there is a laminin G domain with similarities to agrin, laminin, and perlecan (also known as the ALPS domain), followed by a cysteine rich carboxyl terminal region.

The slit protein encoded by the polynucleotide sequence of the present invention has been shown to bind to the transmembrane robo protein with a binding affinity comparable to that observed in other receptor-ligand interactions. The protein has also been shown to be capable of guiding nerve axon projection and neuron migration by repulsion. In addition, the slit protein encoded by SEQ ID NO: 1 has been shown to inhibit the cytokine-induced migration of leukocytes. The ability to inhibit cytokine induced migration of lymphocytes makes the slit protein of the present invention useful in treatment of inflammation caused by the invasion of leukocytes. Therefore, the present invention encompasses methods and compositions for the treatment of inflammation resulting from the infiltration of leukocytes. Leukocytes also play a major role in delayed graft rejection, especially in xenograft rejection (French et al., *Reprod. Fertil. Dev.*, 10:683-696 (1998). Thus, the slit protein of the present invention can be used to prevent graft rejection by preventing the migration of leukocytes into the graft. The inhibition or prevention of graft rejection can be accomplished by the local or systemic administration of the slit protein. Alternatively, when cells are transplanted, either alone or in conjunction with a transplanted organ or tissue, the cells can be transformed so as to secrete the slit protein of the present invention and so prevent damage to the transplant related to leukocyte invasion.

The ability of the slit protein of the present invention to influence cell movement in systems as diverse as the neural and immune systems demonstrates the widespread applicability of the present invention. Thus, the protein encoded by nucleotide sequences of

the present invention can be used to affect the movement or migration of any cell type expressing the robo protein or any other functional receptor for the slit protein of the present invention.

Based on the Drosophila slit sequence, degenerate primers were designed to amplify possible vertebrate slit homologs by the polymerase chain reaction (PCR). One pair of primers allowed isolation of *slit* homologs from Xenopus and chick embryos. A probe made from the PCR fragment of Xenopus *slit* was used to screen a Xenopus embryonic cDNA library, and cDNA clones encoding a full-length Xenopus slit protein were isolated. By low stringency hybridization, cDNAs for *slit* genes were isolated from the mouse. Sequence comparison showed that the Xenopus *slit* gene is an ortholog of the *slit-2* genes of mice (*mslit-2*) and humans (*hslit-2*).

The predicted full-length Xenopus, mouse and human slit proteins were found to share features of the Drosophila slit protein. At the amino (N) terminus, there is a putative signal peptide characteristic of secreted proteins. There are four leucine rich repeats (LRR), each surrounded by an N terminal and a carboxyl (C) terminal flanking region. In Xenopus slit as well as human and mouse slit-2, there are nine epidermal growth factor (EGF) repeats, whereas there are seven EGF repeats in Drosophila slit (Rothberg et al., *Genes Dev.* 4:2169-2187 (1990), Rothberg and Artavanis-Tsakonas, *J. Mol. Biol.* 227:367-370 (1992)). Near the C terminus, there is a laminin G domain with similarities to agrin, laminin, and perlecan (also known as the ALPS domain), followed by a cysteine rich carboxyl terminal region (Rothberg et al., *Genes Dev.* 4:2169-2187 (1990); Rothberg and Artavanis-Tsakonas, *J. Mol. Biol.* 227:367-370 (1992)).

The present invention also involves recombinant polynucleotides comprising the isolated sequence for the vertebrate slit protein along with other sequences. Such recombinant polynucleotides are commonly used as cloning or expression vectors although other uses are possible. A recombinant polynucleotide is one in which polynucleotide sequences of different organisms have been joined together to form a single unit. A cloning vector is a self-replicating DNA molecule that serves to transfer a DNA segment into a host cell. The three most common types of cloning vectors are bacterial plasmids, phages, and other viruses. An expression vector is a cloning vector designed so that a coding sequence inserted at a particular site will be transcribed and translated into a protein.

Both cloning and expression vectors contain nucleotide sequences that allow the vectors to replicate in one or more suitable host cells. In cloning vectors, this sequence is

generally one that enables the vector to replicate independently of the host cell chromosomes, and also includes either origins of replication or autonomously replicating sequences. Various bacterial and viral origins of replication are well known to those skilled in the art and include, but are not limited to the pBR322 plasmid origin, the 2μ plasmid origin, and the SV40, polyoma, adenovirus, VSV and BPV viral origins.

The polynucleotide sequence of the present invention may be used to produce proteins by the use of recombinant expression vectors containing the sequence. Suitable expression vectors include chromosomal, non-chromosomal and synthetic DNA sequences, for example, SV 40 derivatives; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA; and viral DNA such as vaccinia, adenovirus, fowl pox virus, retroviruses, and pseudorabies virus. In addition, any other vector that is replicable and viable in the host may be used.

The nucleotide sequence of interest may be inserted into the vector by a variety of methods. In the most common method the sequence is inserted into an appropriate restriction endonuclease site(s) using procedures commonly known to those skilled in the art and detailed in, for example, Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, (1989) and Ausubel et al., *Short Protocols in Molecular Biology*, 3rd ed., John Wiley & Sons (1995).

In an expression vector, the sequence of interest is operably linked to a suitable expression control sequence or promoter recognized by the host cell to direct mRNA synthesis. Promoters are untranslated sequences located generally 100 to 1000 base pairs (bp) upstream from the start codon of a structural gene that regulate the transcription and translation of nucleic acid sequences under their control. Promoters are generally classified as either inducible or constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in the environment, e.g. the presence or absence of a nutrient or a change in temperature. Constitutive promoters, in contrast, maintain a relatively constant level of transcription. In addition, useful promoters can also confer appropriate cellular and temporal specificity. Such promoters include those that are developmentally-regulated or organelle-, tissue- or cell-specific.

A nucleic acid sequence is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operatively linked to DNA for a polypeptide if it is expressed as a

preprotein which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked sequences are contiguous and, in the case of a secretory leader, contiguous and in reading frame. Linking is achieved by blunt end ligation or ligation at restriction enzyme sites. If suitable restriction sites are not available, then synthetic oligonucleotide adapters or linkers can be used as is known to those skilled in the art (Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, (1989) and Ausubel et al., *Short Protocols in Molecular Biology*, 3rd ed., John Wiley & Sons (1995)).

Common promoters used in expression vectors include, but are not limited to, CMV promoter, LTR or SV40 promoter, the E. coli lac or trp promoters, and the phage lambda PL promoter. Other promoters known to control the expression of genes in prokaryotic or eukaryotic cells can be used and are known to those skilled in the art. Expression vectors may also contain a ribosome binding site for translation initiation, and a transcription terminator. The vector may also contain sequences useful for the amplification of gene expression.

Expression and cloning vectors can and usually do contain a selection gene or selection marker. Typically, this gene encodes a protein necessary for the survival or growth of the host cell transformed with the vector. Examples of suitable markers include dihydrofolate reductase (DHFR) or neomycin or hygromycin B resistance for eukaryotic cells and tetracycline, ampicillin, or kanamycin resistance for *E. coli*.

In addition, expression vectors can also contain marker sequences operatively linked to a nucleotide sequence for a protein that encode an additional protein used as a marker. The result is a hybrid or fusion protein comprising two linked and different proteins. The marker protein can provide, for example, an immunological or enzymatic marker for the recombinant protein produced by the expression vector. In a preferred embodiment of the present invention, alkaline phosphatase (AP), green fluorescence protein (GFP), myc, histidine tag (His) and hemagglutinin (HA) are used as markers.

Additionally, the end of the polynucleotide can be modified by the addition of a sequence encoding an amino acid sequence useful for purification of the protein produced by affinity chromatography. Various methods have been devised for the addition of such affinity purification moieties to proteins. Representative examples can be found in U.S. Patent Nos.

4,703,004, 4,782,137, 4,845,341, 5,935,824, and 5,594,115. Any method known in the art for the addition of nucleotide sequences encoding purification moieties can be used for example those contained in Innis et al., *PCR Protocols*, Academic Press (1990) and Sambrook et al., *Molecular Cloning*, 2nd ed., Cold Spring Harbor Laboratory Press (1989).

More particularly, the present invention includes recombinant constructs comprising the isolated polynucleotide sequence of the present invention. The constructs can include a vector, such as a plasmid or viral vector, into which the sequence of the present invention has been inserted, either in the forward or reverse orientation. The recombinant construct further comprises regulatory sequences, including for example, a promoter operatively linked to the sequence. Large numbers of suitable vectors and promoters are known to those skilled in the art and are commercially available. In one preferred embodiment, the pCS2+, the pCEP4 (Invitrogen) and the pIRESneo (Clontech) vectors are used. It will be understood by those skilled in the art, however, that other plasmids or vectors may be used as long as they are replicable and viable or expressing the encoded protein in the host.

The polynucleotide sequences of the present invention can also be part of an expression cassette that at a minimum comprises, operably linked in the 5' to 3' direction, a promoter, a polynucleotide of the present invention, and a transcriptional termination signal sequence functional in a host cell. The promoter can be of any of the types discussed herein, for example, a tissue specific promoter, a developmentally regulated promoter, an organelle specific promoter, etc. The expression cassette can further comprise an operably linked targeting sequence, transit or secretion peptide coding region capable of directing transport of the protein produced. The expression cassette can also further comprise a nucleotide sequence encoding a selectable marker and a purification moiety.

A further embodiment of the present invention relates to transformed host cells containing the constructs comprising the polynucleotide sequence of the present invention. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell such as an insect cell or a yeast cell, or the host can be a prokaryotic cell such as a bacterial cell. Introduction of the construct into the host cell can be accomplished by a variety of methods including calcium phosphate transfection, DEAE-dextran mediated transfection, Polybrene mediated transfection, protoplast fusion, liposome mediated transfection, direct microinjection into the nuclei, biolistic (gene gun) devices, scrape loading, and electroporation.

The present invention also relates to proteins encoded by the isolated polynucleotide. As used herein the term protein includes fragments, analogs and derivatives of the slit protein. The terms "fragment," "derivative" and "analog" as used herein mean a polypeptide that retains essentially the same biological function or activity as the slit protein encoded by the sequence of the present invention. For example, an analog includes a proprotein which can be cleaved to produce an active mature protein. The protein of the present invention can be a natural protein, a recombinant protein or a synthetic protein or a polypeptide.

Those of ordinary skill in the art are aware that modifications in the amino acid sequence of a peptide, polypeptide, or protein can result in equivalent, or possibly improved, second generation peptides, etc., that display equivalent or superior functional characteristics when compared to the original amino acid sequence. The present invention accordingly encompasses such modified amino acid sequences. Alterations can include amino acid insertions, deletions, substitutions, truncations, fusions, shuffling of subunit sequences, and the like, provided that the peptide sequences produced by such modifications have substantially the same functional properties as the naturally occurring counterpart sequences disclosed herein. Biological activity or function can be determined by, for example, the ability of the protein to guide cell migration (Example 10), or by the ability to repulse axon projection (Example 8); by the ability of the protein to specifically bind to the robo protein (Example 7) or by the ability to alter cytokine induced migration of leukocytes (Example 11).

One factor that can be considered in making such changes is the hydropathic index of amino acids. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein has been discussed by Kyte and Doolittle (*J. Mol. Biol.*, 157: 105-132, 1982). It is accepted that the relative hydropathic character of amino acids contributes to the secondary structure of the resultant protein. This, in turn, affects the interaction of the protein with molecules such as enzymes, substrates, receptors, DNA, antibodies, antigens, etc.

Based on its hydrophobicity and charge characteristics, each amino acid has been assigned a hydropathic index as follows: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate/glutamine/aspartate/asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

As is known in the art, certain amino acids in a peptide or protein can be substituted for other amino acids having a similar hydropathic index or score and produce a resultant

peptide or protein having similar biological activity, i.e., which still retains biological functionality. In making such changes, it is preferable that amino acids having hydropathic indices within ±2 are substituted for one another. More preferred substitutions are those wherein the amino acids have hydropathic indices within ±1. Most preferred substitutions are those wherein the amino acids have hydropathic indices within ±0.5.

Like amino acids can also be substituted on the basis of hydrophilicity. U.S. Patent No. 4,554,101 discloses that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. The following hydrophilicity values have been assigned to amino acids: arginine/lysine (+3.0); aspartate/glutamate (+3.0 ±1); serine (+0.3); asparagine/glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ±1); alanine/histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine/isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). Thus, one amino acid in a peptide, polypeptide, or protein can be substituted by another amino acid having a similar hydrophilicity score and still produce a resultant protein having similar biological activity, i.e., still retaining correct biological function. In making such changes, amino acids having hydropathic indices within ±2 are preferably substituted for one another, those within ±1 are more preferred, and those within ±0.5 are most preferred.

As outlined above, amino acid substitutions in the peptides of the present invention can be based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, etc. Exemplary substitutions that take various of the foregoing characteristics into consideration in order to produce conservative amino acid changes resulting in silent changes within the present peptides, etc., can be selected from other members of the class to which the naturally occurring amino acid belongs. Amino acids can be divided into the following four groups: (1) acidic amino acids; (2) basic amino acids; (3) neutral polar amino acids; and (4) neutral non-polar amino acids. Representative amino acids within these various groups include, but are not limited to: (1) acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; (2) basic (positively charged) amino acids such as arginine, histidine, and lysine; (3) neutral polar amino acids such as glycine, serine, threonine, cysteine, cystine, tyrosine, asparagine, and glutamine; and (4) neutral non-polar amino acids such as alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. It should be noted that changes which

are not expected to be advantageous can also be useful if these result in the production of functional sequences.

The fragment, derivative or analog of the proteins encoded by the polynucleotide sequence of the present invention may be, for example and without limitation, (i) one in which one or more amino acid residues are substituted with a conserved or non-conserved amino acid residue, and such substituted amino acid residue may or may not be one encoded by the genetic code; (ii) one in which one or more of the amino acid residues includes a substituent group; (iii) one in which the mature protein is fused to another compound such as a compound to increase the half-life of the protein; (iv) one in which additional amino acids are fused to the protein to aid in purification or in detection and identification; or (v) one in which additional amino acid residues are fused to the protein to aid in modifying tissue distribution or localization of the protein to certain locations such as the cell membrane or extracellular compartments.

The term protein also includes forms of the slit protein to which one or more substituent groups have been added. A substituent is an atom or group of atoms that is introduced into a molecule by replacement of another atom or group of atoms. Such groups include, but are not limited to lipids, phosphate groups, sugars and carbohydrates. Thus, the term protein includes, for example, lipoproteins, glycoproteins, phosphoproteins and phospholipoproteins.

The present invention also includes methods for the production of the slit protein from cells transformed with the polynucleotide sequence of the present invention. Proteins can be expressed in mammalian cells, plant cells, insect cells, yeast, bacteria, bacteriophage, or other appropriate host cells. Host cells are genetically transformed to produce the protein of interest by introduction of an expression vector containing the nucleic acid sequence of interest. The characteristics of suitable cloning vectors and the methods for their introduction into host cells have been previously discussed. Alternatively, cell-free translation systems can also be employed using RNA derived from the DNA of interest. Methods for cell free translation are known to those skilled in the art. (Davis et al., *Basic Methods in Molecular Biology*, Elsevier Science Publishing (1986); Ausubel et al., *Short Protocols in Molecular Biology*, 2nd Ed., John Wiley & Sons (1992)). In the preferred embodiment, host cells are HEK 293 cells or 293T cells (American Type Culture Collection).

Host cells are grown under appropriate conditions to a suitable cell density. If the sequence of interest is operably linked to an inducible promoter, the appropriate

environmental alteration is made to induce expression. If the protein accumulates in the host cell, the cells are harvested by, for example, centrifugation or filtration. The cells are then disrupted by physical or chemical means to release the protein into the cell extract from which the protein can be purified. If the host cells secrete the protein into the medium, the cells and medium are separated and the medium retained for purification of the protein.

Larger quantities of protein can be obtained from cells carrying amplified copies of the sequence of interest. In this method, the sequence is contained in a vector that carries a selectable marker and transfected into the host cell or the selectable marker is co-transfected into the host cell along with the sequence of interest. Lines of host cells are then selected in which the number of copies of the sequence have been amplified. A number of suitable selectable markers will be readily apparent to those skilled in the art. For example, the dihydrofolate reductase (DHFR) marker is widely used for co-amplification. Exerting selection pressure on host cells by increasing concentrations of methotrexate can result in cells that carry up to 1000 copies of the DHFR gene.

Proteins recovered can be purified by a variety of commonly used methods, including, but not limited to, ammonium sulfate precipitation, immuno precipitation, ethanol or acetone precipitation, acid extraction, ion exchange chromatography, size exclusion chromatography, affinity chromatography, high performance liquid chromatography, electrophoresis, and ultra filtration. If required, protein refolding systems can be used to complete the configuration of the protein.

Practice of this invention also includes the therapeutic use of the protein encoded by the polynucleotide sequence and its inclusion in pharmaceutical compounds. A greater understanding of the mechanisms directing nerve axon growth is critical to the application of methods for restoring nerve tracts after, for example, spinal cord injury. Applicants have shown that one of the biological functions of the protein encoded by the polynucleotide sequence of the present invention is direction of axon growth by directing axons away from the source of the protein (repulsive guidance). Another biological function of the protein is the guidance of migrating cells by repulsing the migrating cells from the source of the slit protein. As used herein the terms repulsing, repulsive and repulsive guidance mean directing the projection (growth) of axons or the migration of cells away from the source of the slit protein. A repulsive amount means an amount of slit protein sufficient to direct growing axons or migrating cells away from the source of the slit protein. In addition, by alteration of conditions, slit protein may act as an attractive guidance cue. Changes in condition that may

alter slit protein function include, but are not limited to, changes in the cell type or developmental stage of the cell or changes in the concentration of cAMP, cGMP and other cofactors.

In vitro, transformed cells secreting the slit protein can be used to determine which nerve tracts are guided to their target tissue by repulsive forces. For example, transformed cells can be enclosed within a collagen matrix along with explants of neural tissue. The effect on axon growth from the explant on the sides proximal and distal to the transformed cells can then be determined. Alternatively, transformed cells can be enclosed in microspheres constructed of material which allows diffusion of the secreted products of the cells. In another alternative, the protein can be delivered to explants by the use of mini pumps to supply a steady source of the protein in the vicinity of the explant.

Repair of spinal cord injuries requires not only that the nervous tissue be stimulated to grow new axons, but in addition, the new axons must be directed to the proper site within the nervous system to reestablish the previous nerve tracts. Recently, Xu et al., *Eur. J. Neurosci.* 11:1723-1740 (1999), have reported the regrowth of spinal neurons by the use of a mini chamber implanted within the spinal cord. Key to axon regrowth is the presence of Schwann cells within the mini-chamber. Although axons can be induced to regenerate through the use of the chamber, the growth is not directed. It is believed that by seeding cells that have been transformed with the nucleotide sequence of the present invention and that secrete the protein encoded by the sequence, the regenerating axons can be directed to a specific location in the distal spinal cord.

The slit protein of the present invention is also useful for the treatment of conditions involving the migration of leukocytes. As reviewed above, leukocytes are thought to be involved in a number of conditions. Thus, the ability to inhibit leukocyte migration into particular organs or tissues would be beneficial in the treatment of numerous diseases, conditions or disorders. Representative conditions in which inhibition of leukocyte migration by administration of the slit proteins encoded by the nucleotide sequence of the present invention include, but are not limited to, asthma, arthritis, glomerulonephritis, cystic fibrosis, ulcerative colitis, Crohn's disease, multiple sclerosis, allergic encephalomyelitis, Alzheimer's disease, coronary artery restenosis, and any other condition which may be alleviated by an inhibition of migration of leukocytes into the affected tissue or organ.

In addition, the slit protein of the present invention is useful for the prevention or inhibition of graft rejection associated with leukocytes, especially delayed rejection and more

particularly delayed rejection of xenografts. The slit protein acts to lessen or prevent graft rejection by inhibiting the migration of leukocytes into the graft. This can be accomplished by the systemic or local administration of the protein. For example, local administration can be accomplished by transplantation, along with the tissue or organ, of a mini-pump to slowly release the slit protein over time. Alternatively, cells can be transformed with the polynucleotides of the present invention such that they secrete slit protein. When the objective is to transplant the cells themselves, such as the insulin producing cells of the pancreas, the secretion of the slit protein can serve to protect the cells. When organs are being transplanted, transformed endothelial cells of the blood vessels can be used to limit leukocyte invasion. Other methods of using slit proteins to inhibit graft rejection will be apparent to those skilled in the art and are within the scope of the present invention.

Without being limited by theory, the protein of the present invention is thought to act by binding to the robo protein. Thus, the present invention can be applied to direct the growth or migration of any cell type expressing the robo protein or other functional slit protein receptor on its surface, for example to direct the growth of regenerating tissues or organs. In addition, the ability of proteins encoded by the nucleotide sequence to inhibit and/or redirect cell movement, has use in preventing migration of malignant cells which express the robo protein or other functional slit protein receptors.

Use of the slit protein is not limited to cells that naturally express the *robo* gene or genes for other functional slit protein receptors, but includes cells into which the *robo* gene or genes for other functional slit protein receptors have been introduced through recombinant DNA technology. For example, cells could be designed that produce both a therapeutic agent and also express the *robo* gene or other functional slit protein receptor gene. Such cells could then be transplanted into the body to a location near where the therapeutic agent was needed. By using the slit protein, the cells producing the agent could be directed to a location where the therapeutic agent was needed. For example, cells producing a factor necessary to stimulate nerve regeneration could be directed to a specific site where nerve damage has occurred. In another example, the slit protein could be used to prevent the migration of cells. In this use, cells transplanted to a location in the body, for example, could be prevented from migrating by local administration of the slit protein by, for example, a transplantable mini pump or by co-transplantation of cells secreting the slit protein. This method can also be applied to inhibiting the migration of malignant cells expressing the robo protein. By local administration of the slit protein, the metastasis of the malignant cells can be prevented or

inhibited. Examples of malignant cells that express the robo protein include, but are not limited to, neuroblastoma cells and glioblastoma cells.

The slit protein encoded by the polynucleotide of the present invention has been shown to be effective in blocking the action of RANTES and SDF-1. Receptors for chemokines, including RANTES and SDF-1, have been reported to play a role in as recognition sites for HIV infection (See, Baggiolini and Moser, *J. Exp. Med.*, 186:1189-1191, 1997). Antagonists of chemokines have also been reported as blocking HIV infection (Simmons et al., *Science*, 276:276-279, 1997; Arenzana-Seisdedos et al., *Nature*, 383:400, 1996). Thus, the slit protein of the present invention can be used to limit HIV infection of cells *in vitro* or *in vivo*. When administered *in vivo*, the amount administered will vary with such factors as the size, age, sex, health and virus load of the patient, as well other factors well known in the art.

The slit proteins of the present invention can be formulated as pharmaceutical compositions. Such compositions can be administered orally, parenterally, by inhalation spray, rectally, intradermally, transdermally, or topically in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants, and vehicles as desired. Topical administration may also involve the use of transdermal administration such as transdermal patches or iontophoresis devices. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, or intrasternal injection, or infusion techniques. Formulation of drugs is discussed in, for example, Hoover, John E., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pennsylvania (1975), and Liberman, H.A. and Lachman, L., Eds., *Pharmaceutical Dosage Forms*, Marcel Decker, New York, N.Y. (1980).

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions, can be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed, including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are useful in the preparation of injectables. Dimethyl acetamide, surfactants including ionic and non-ionic detergents, and

polyethylene glycols can be used. Mixtures of solvents and wetting agents such as those discussed above are also useful.

Suppositories for rectal administration of the compounds discussed herein can be prepared by mixing the active agent with a suitable non-irritating excipient such as cocoa butter, synthetic mono-, di-, or triglycerides, fatty acids, or polyethylene glycols which are solid at ordinary temperatures but liquid at the rectal temperature, and which will therefore melt in the rectum and release the drug.

Solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the compounds of this invention are ordinarily combined with one or more adjuvants appropriate to the indicated route of administration. If administered *per os*, the compounds can be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, cellulose alkyl esters, talc, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfuric acids, gelatin, acacia gum, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and then tableted or encapsulated for convenient administration. Such capsules or tablets can contain a controlled-release formulation as can be provided in a dispersion of active compound in hydroxypropylmethyl cellulose. In the case of capsules, tablets, and pills, the dosage forms can also comprise buffering agents such as sodium citrate, or magnesium or calcium carbonate or bicarbonate. Tablets and pills can additionally be prepared with enteric coatings.

For therapeutic purposes, formulations for parenteral administration can be in the form of aqueous or non-aqueous isotonic sterile injection solutions or suspensions. These solutions and suspensions can be prepared from sterile powders or granules having one or more of the carriers or diluents mentioned for use in the formulations for oral administration. The compounds can be dissolved in water, polyethylene glycol, propylene glycol, ethanol, corn oil, cottonseed oil, peanut oil, sesame oil, benzyl alcohol, sodium chloride, and/or various buffers. Other adjuvants and modes of administration are well and widely known in the pharmaceutical art.

Liquid dosage forms for oral administration can include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water. Such compositions can also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

The amount of protein that can be combined with the carrier materials to produce a single dosage form will vary depending upon the patient and the particular mode of administration.

Slit protein or slit protein derivatives can also be use to stimulate cell proliferation. Rates of cell proliferation can be increased, for example, by transforming cells with vectors containing the polynucleotide encoding the slit protein. Alternatively, cell proliferation can be increased by treating cells with slit protein, either by addition of slit protein to the culture medium or by conditioning the medium with cells secreting the slit protein. Conditioned medium is obtained by culturing cells secreting the slit protein in the medium, harvesting the medium, and using the medium to culture additional cells. *In vivo*, slit can be administered locally or systemically to stimulate cell proliferation by any of the methods or routes described previously, by transplanting transformed cells that secrete the slit protein, or by the use of a transplantable mini pump that delivers slit protein to the desired location. *In vivo* the ability of slit to stimulate cell proliferation can be used, for example, to aid in wound healing or repair, or in organ regeneration such as lung, liver and kidney regeneration following injury or disease. The exact amount of slit administered will vary based on the size, age, and sex of the animal, the route and method of administration, the cell type to be stimulated, the amount of cell proliferation desired, and other factors well known in the art.

The slit protein can also be used to reduce or eliminate serum in cell culture medium. Serum, for example fetal bovine serum, is commonly added to cell culture media to promote cell growth. The addition of serum to cell culture media is undesirable because the composition of serum varies from batch to batch. Thus, the use of serum adds unknown variables to experiments using cell culture which can complicate analysis and cause inconsistent results. The need for serum in culture medium can be reduced or eliminated by transforming cells with polynucleotides encoding the slit protein. Without being bound by theory, it is believed that the need for serum is reduced or eliminated because the transformed cells secrete slit protein into the culture medium. Thus, it is also believed that the need for serum in culture medium can be eliminated or reduced by the addition of slit protein to the culture medium. As used herein, reduced serum culture medium means culture medium in which the amount of serum normally used is decreased by at least 25% due to the addition of slit protein or slit protein derivatives.

Example 1

Isolation of Vertebrate Slit cDNA

Degenerate primers corresponding to NPFNCNC (SEQ ID NO: 4) and CETNIDDC (SEQ ID NO: 5) of Drosophila slit protein (amino acid residues 652-658 and 981-988 respectively) were used to clone fragments of Xenopus *slit* cDNA by PCR. A cDNA library was constructed using the BKCMV vector (Strategene) with mRNAs extracted from stage 17 Xenopus embryos. The PCR fragment of Xenopus *slit* was used to screen the cDNA library to obtain full-length Xenopus *slit* cDNA clones. Human slit-2 cDNA was obtained using PCR with cDNA prepared from human fetal brain mRNA (Clontech) and primers corresponding to MRGVGW (amino acid residues 1-6) and CTRCVS (the last six amino acid residues in the coding region). The full-length Xenopus *slit* and human *slit-2* cDNAs were sequenced using an ABI 373A automatic sequencer (Applied Biosystems) in accordance with the manufacturer's protocol and the amino acid sequence deduced from the nucleotide sequence. The human slit-2 cDNA isolated differs from the published huamn slit-2 sequence (Genbank accession # AF055585) (SEQ ID NO: 3) by an insertion of eight amino acid residues (AKEQYFIP) (SEQ ID NO: 6) between residue 479 and 480. This is possibly a result of alternative splicing as predicted from sequence analysis. Methods for the construction and screening of cDNA libraries are well known to those skilled in the art and can be found, for example in Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Press, (1989) and Ausubel et al., *Short Protocols in Molecular Biology*, 2nd Ed., John Wiley & Sons (1992).

The nucleotide sequence for the Xenopus slit cDNA is given in Figure 1. The cDNA sequence consists of 5513 bases and exhibits 59% sequence identity with the murine slit-2 sequence. The deduced amino acid sequence encoded by the cDNA is given in Figure 2. The protein exhibits 87% amino acid identity with the mouse slit-2 protein, 85% identity with human slit-2 protein, 63% identity with the mouse slit-1 and mouse slit-3 proteins, 83% identity with chicken slit protein, 67% with the rat slit protein rMEGF5, 63% with the rat slit protein rMEGF4 (Nakayama et al., *Genomics* 51:27-34 (1998)), and 40% identity with the Drosophila slit protein. At the amino (N) terminus, there is a putative signal peptide characteristic of secreted proteins. There are four leucine rich repeats (LRR), each surrounded by an N terminal and a carboxyl (C) terminal flanking region. In Xenopus slit, as with mouse and human slit-2, there are nine epidermal growth factor (EGF) repeats, whereas

there are seven EGF repeats in Drosophila slit (Rothberg et al., *Genes Dev.* 4:2169-2187 (1990), Rothberg and Artavanis-Tsakonas, *J. Mol. Biol.* 227:367-370 (1992)). Near the C terminus, there is a laminin G domain with similarities to agrin, laminin, and perlecan (also known as the ALPS domain), followed by a cysteine rich carboxyl terminal region (Rothberg et al., *Genes Dev.* 4:2169-2187 (1990), Rothberg and Artavanis-Tsakonas, *J. Mol. Biol.* 227:367-370 (1992)).

Example 2

Plasmid Construction

The coding region for full-length Xenopus slit was inserted in-frame into the pCS2+ vector containing a six-myc epitope tag or containing the secreted form of alkaline phosphatase to obtain slit-myc or slit-AP, respectively. The coding region for full-length human slit-2 was inserted into the pCS2+ vector containing the myc tag to obtain hslit-myc expressing plasmid. In both cases, slit was at the N terminal portion of the fusion proteins. To express robo as an epitope tagged protein, the rat robo1 coding region was obtained by PCR using rat spinal cord cDNA and was then inserted into pCS2+ vector containing a hemagglutinin (HA) epitope. The HA epitope was at the C terminus of robo-1. Plasmids were also constructed to express different fragments of Xenopus slit, human slit-2 or robo. The full-length or fragments of coding regions of slit and robo-1 in the plasmids were verified by sequencing. Before being used in transfection experiments, individual plasmids were also tested for expression of the corresponding proteins by coupled in vitro transcription-translation (Promega). The plasmid construction was carried out following standard protocols described in Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Press, (1989) and Ausubel et al., *Short Protocols in Molecular Biology*, 2nd Ed., John Wiley & Sons (1992).

Example 3

Cell Culture, Transfection and Immunoprecipitation

To directly test the possible ligand-receptor relationship between slit and robo, cells were transformed to express the slit and robo fusion proteins and the interactions of the proteins examined by immunoprecipitation and Western blotting.

HEK 293 cells or 293T cells (both from American Type Culture Collection) were maintained in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) (Gibco). Cells were grown to 70% confluence on 2-10 cm tissue culture dishes and transfected with 5-25 μg of plasmid DNA per plate using lipofectin or lipofectamine (Life Technology), commercially available liposome preparations or by electroporation according to the manufacturer's instructions. Alternatively, cells were transfected using calcium phosphate for 6 - 24 hours. GFP-pGL, a plasmid expressing the green fluorescent protein (GFP) (Life Technology), was used in most of the transfection experiments to monitor transfection efficiency.

For co-immunoprecipitation, plasmids encoding slit-myc, slit-AP, or robo-HA or control vector plasmids were transfected into HEK or 293T cells as described above. Conditioned media containing slit-myc or slit-AP proteins from the transfected cells were collected 72 to 96 hrs after transfection and concentrated using a Biomax-100K ultrafree-15 filter (Millipore). Robo-HA containing cell lysates or control lysates were prepared with lysis buffer (0.5% NP-40, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 50mM NaF, 1mM Na_3VO_4, 1mM DTT, 1mM phenylmethylsulfonyl fluoride, 25μg/ml leupeptin, 25μg/ml aprotinin, 150μg/ml benzamidine). Conditioned media containing slit-myc or slit-AP were mixed with lysates from robo-HA or control cells. Immunoprecipitation was carried out as described in Kopan et al., *Proc. Natl. Acad. Sci. USA* 93:1683-1688 (1996) using anti-myc (Babco) or anti-AP (Sigma) antibodies. Precipitated proteins were then detected after Western blotting by anti-HA (Babco) with enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

Following incubation of slit-myc medium with robo-HA cell lysate, slit-myc could be immunoprecipitated with anti-HA antibody. Conversely, robo-HA could be precipitated from the slit containing medium mixed with robo containing cell-lysate by anti-myc antibodies. These results provide evidence that the slit protein of the present invention binds to robo.

Example 4

Cell Surface Binding and Immunocytochemistry

To confirm that the binding of the slit and robo proteins was not confined to cell-free systems, but also occurred at the cell surface, the binding of slit-AP to cells expressing the robo-HA protein was determined.

HEK 293 cells grown in 10 cm dishes were transfected with robo-HA or vector plasmids as previously described. Approximately 30-48 hours after transfection, cells were suspended by pipetting up and down several times and then seeded onto 6- well or 24-well dishes to 50% confluence. Cells were grown for another 12 to 18 hrs before incubation with either slit-AP fusion protein or lunatic fringe alkaline phosphatase (lFng-AP) fusion protein conditioned media containing similar amounts of AP activity (approximately 530 OD/ml/hr). Lunatic fringe is a vertebrate signaling protein found in Xenopus used here as a negative control. The lFng-AP protein was made by the method of Wu et al., *Science* 273:355-358 (1996).

Cells were incubated for 1 hr with the slit-AP containing media or the control media followed by three to four washes in HBHA buffer (Hank's balanced salt solution, 0.5mg/ml BSA, 20mM HEPES, pH 7.0), and then fixed for 30 seconds in acetone-formaldehyde fixative (60% acetone, 3% formaldehyde, 20mM HEPES pH 7.0). Fixed cells were washed three times in HBS (150mM NaCl, 20mM HEPES, pH7.0) and incubated at 65°C for 10 min to inactivate the endogenous cellular phosphatase activity. AP staining buffer (100mM Tris, pH9.5, 50mM MgCl2, 100mM NaCl, 0.1% Tween 20, 0.17mg/ml 5-bromo-1-chloro-3-indoxyl phosphate (BCIP), 0.33mg/ml nitroblue tetrazolium) was used to detect slit-AP or lFng-AP bound at the cell surface. Following three washes, robo-HA expression on AP positive cells was confirmed by the double antibody staining method by treatment with mouse anti-HA and anti-mouse immunoglobulin conjugated to cyanine dye 3 (Cy3, Jackson Immunoresearch). GFP expression indicated similar transfection efficiencies in vector and robo-HA transfected cells.

Slit-AP binding was found to correlate with robo-HA expression providing additional evidence that slit binds to robo on the cell surface. The lunatic fringe-AP fusion protein did not bind to the robo transfected cells. These results indicate that slit specifically binds to cell surface robo proteins.

Example 5

Production of Cell Lines Exhibiting Stable Expression of Slit or Robo Proteins

Several stable cell lines were made using a human embryonic kidney (HEK) cell line (American Tissue Culture Center Accession Number CRL-1573). Vector control lines were made by transfection with the vector plasmid pIRESneo (Clontech) or pCEP4 (Invitrogen).

Xslit-myc line and hslit-myc line express full-length Xenopus Slit and human slit2 proteins, respectively. Stable cell lines were also prepared to express different regions of human slit-2 protein including N890 (amino-terminal 890 amino acid residues), N1076 (amino-terminal 1076 amino acid residues) and C1 (nucleotide sequence encoding carboxyl-terminal 892-1529 amino acid residues fused to the sequence encoding the predicted signal peptide). Stable cell lines were prepared to express different regions of Xenopus slit protein including N882 (amino-terminal 882 amino acid residues), and C1 (nucleotide sequence encoding carboxyl-terminal 883-1530 amino acid residues fused to the sequence encoding the predicted signal peptide). Cells expressing full-length Xenopus Slit and human slit2 proteins also express proteolytic fragments, similar to that reported by Wang, K-H et al. (*Cell* 96:771-784, 1999). In addition, human slit2 stable cell lines expressing individual mutations at amino acid residue L538S and R172G/C1358Y were made. These two mutant slit-2 stable lines have altered pattern in proteolytic cleavage compared to the wild-type slit-2 cells.

Stable cell lines were also established expressing the full-length Robo and Robo-N (amino-terminal 730 amino acid residues) as proteins tagged with a hemagglutinin (HA) tag at their carboxyl termini. The full-length Robo and Robo-N plasmids for making stable cell lines were constructed using the vector modified from pIRESneo (Clontech). Another Robo-HA stable line, Robo-HA-hygB, was also constructed using the pCEP4 vector (Invitrogen).

Linearized or circular plasmids encoding Slit-myc and Robo-HA (full-length or derivatives) and their corresponding vector controls were transfected into HEK cells. Antibiotics were added 24 to 48 hours after transfection and selection was carried out for three to five weeks with the media changed every two to three days. G418 (200-500 μg/ml) (Life Technology) was used to select for Slit-myc and Robo-HA (full-length or derivatives) stable lines. Hygromycin B (100-300 μg/ml) (Sigma) was used to select for Robo-HA-hygB stable lines. Stable cell lines expressing Slit-myc and Robo-HA were obtained after isolating individual colonies and testing for protein expression by both Western blots and immunocytochemical staining (with anti-myc or anti-HA antibodies, respectively).

Example 6

Purification of Slit Proteins and Their Derivatives

To purify 220Kd full-length Slit protein, cells stably expressing Slit were grown to 90-95% confluence, treated with trypsin and plated at 30-40% confluence. Cells were then grown for 20-36 hours before collecting the media. To purify slit fragments, Slit stable cells

expressing corresponding slit fragments [N890, N1076, C1 fragments] or carboxyl-terminal fragment cleavage product from the full-length slit were grow to confluence and media were collected 2-5 days after plating cells.

The Slit protein (full-length or derivatives) containing media was adjusted to pH 5.5-6.5 with acetic acid and then loaded onto a SP-sepharose ion exchange chromatography column (Pharmacia) that had been equilibrated with 10 bed volumes of PBS, pH 5.5-6.5. The column was then washed with 10 bed volumes of PBS, pH 5.5-6.5. Slit protein was eluted with a 0-1.0 M NaCl gradient. The full-length Slit protein was usually eluted out at 0.4 to 0.6 M NaCl and the N890, N1076, and C1 fragments usually eluted at 0.2 to 0.5 M NaCl. Fractions were collected and examined by Western blotting and silver staining to determine which fractions contained Slit protein of the desired purity. The desired fractions were combined for further purification or concentration. Further purification was achieved with immuno-affinity chromatography by 9E10-agarose (Babco) or Superose (Pharmacia) gel filtration. For immuno-affinity chromatography, the slit containing fractions were adjusted to pH 7.5 and loaded onto a 9E10-agarose column (Babco). The media were passed through the column three times and the column was then washed with 10 bed volumes of PBS. Slit protein was eluted with 0.1 M Glycine pH 2.9 and the pH of the protein eluted was adjusted to 7.5 with Tris-HCl by adding 1/10 volume of 1 M Tris HCl, pH 7.5. Gel filtration was carried out using a Superose column (Pharmacia) according to the manufacturer's instructions. If necessary, Slit protein preparations were further concentrated by using polyethylene glycol (PEG) (Sigma) or a filter device (Millipore). The slit protein and its derivatives can be purified with other variations of ion exchange chromatography with gel filtration and/or affinity chromatography. The purity of proteins was determined by silver staining.

Example 7

Quantitative Assay of Slit-AP Binding to Robo Expressing Cells

In order to determine if the interaction of the slit and robo proteins exhibited a binding affinity similar to that observed between other soluble and cell surface proteins, the binding affinity was determined. The method used has been published previously (Flanagan and Leder, *Cell* 63:185-194 (1990); Cheng and Flanagan, *Cell* 79:157-168 (1994); Keino-Masu et al., *Cell* 87:175-185 (1996); Leonardo et al., *Nature* 386:833-838 (1997)) and so will only be briefly described herein.

Stable cell lines expressing robo-HA or the vector were seeded in 24 well-culture dishes pre-coated with 100mg/ml polylysine. Cells were grown to 95% confluence and then incubated at 37°C for 1hr in 200µl/well of concentrated, conditioned media containing different concentrations of slit-AP with the highest level at approximately 3000 OD/ml/hr of AP activity. Media were collected to assay for the AP activity as free slit-AP. Cells were washed 4 times with the culture medium without slit-AP. Cells were then lysed using a lysis buffer containing 50mM Tris, pH 8 and 1% Triton-X 100. Cell lysates were cleared by centrifugation for 10 min. Samples were heated at 65°C for 10 min to inactivate the endogenous cellular phosphatase activity. AP activity was assayed by adding equal volumes of 2X AP buffer (2M diethanolamine, pH9.8, 1mM MgCl2, 20mM homoarginine, 12 mM p-nitro-phenyl phosphate) to cell lysates in 96-well flat-bottom microtiter plates. Incubation was carried out at room temperature and AP activity was determined at 405nm. The concentration of slit-AP fusion protein in the conditioned media was estimated by comparison with the purified human placenta alkaline phosphatase (HuPAP) (Sigma) both in AP activity assays and in Western blots using anti-AP antibody (Genzyme).

An apparent dissociation constant (K_D) of 2.75 nM was estimated from a binding curve and is comparable to other ligand-receptor interactions (Flanagan and Leder, *Cell* 63:185-194 (1990); Cheng and Flanagan, *Cell* 79:157-168 (1994); Keino-Masu et al., *Cell* 87:175-185 (1996); Leonardo et al., *Nature* 386:833-838 (1997)). These results indicated that soluble slit proteins bind with high affinity to robo cell surface proteins.

Example 8

Effect of Slit protein on Axon Outgrowth from Olfactory Bulb Explants.

To determine if slit plays a role in axon guidance, olfactory bulb explants were isolated from chick embryos and co-cultured with either HEK cells stably transfected with either slit-myc or vector plasmid.

Preparation of rat tail collagen and explant culture in collagen gel matrices were carried out according to the protocol described in Guthrie and Lumsden, *Neuroprotocols* 4:116-120 (1994). Cell aggregates were prepared by the hanging-drop method (Fan and Tessier-Lavigne, *Cell* 79:1175-1186 (1994)).

The olfactory bulb explant assay was performed according to Pini, *Science* 261:95-98 (1993); Keynes et al., *Neuron* 18:889-897 (1997). Briefly, stage 33 or 34 chick embryos were dissected out in Tyrode's solution. Olfactory bulbs were removed and stored in L15 medium with 5% horse serum (GIBCO) on ice for at least 30 min. Olfactory bulb explants were trimmed to 200-300 μm. Olfactory bulb explants and cell aggregates were transferred onto a collagen pad and covered with collagen. The distance between the olfactory bulb explants and the cell aggregates ranged from 100 to 400 μm. After collagen gel matrices solidified, DMEM with 10% FCS and 100 μg/ml of penicillin and streptomycin was added. Explants and cells were co-cultured at 37^0C with 5% CO_2 and the effects of slit on olfactory bulb axons were visible 10-24 hours after culturing. Explants were fixed after about 12 or 24 hours of co-culture. TuJ1 antibody was used in immunocytochemistry to visualize neuronal processes. TuJ1 is a monoclonal antibody that recognizes an epitope unique to neuronal-associated class III beta-tubulin isotypes (Caccamo et al., *Lab. Invest.* 60:390-398 (1989)).

Quantification of the axon projections from the olfactory bulb explants was carried out according to the method of Keynes et al. *Neuron* 18:889-897 (1997). A score of 0 was given if there was no or very few axons growing in the proximal quadrant; a score of 2 was given if there were few axons in the proximal quadrant with strong asymmetry when compared with the distal quadrant; a score of 4 was given if there was greater outgrowth in the proximal quadrant, with axons in the proximal quadrant still more than 50 μm from the cell aggregates, and strong asymmetry between the distal and proximal quadrants; a score of 6 was given if axons in the proximal quadrant were growing within less than 50 μm from the cell aggregates, still with asymmetry between the distal and proximal quadrants; a score of 8 was given if axons were contacting the cell aggregates, but still with detectable asymmetry; and a score of 10 was given if axons had grown over the cell aggregates and there was no asymmetry between the proximal and distal quadrants.

When olfactory bulb explants were co-cultured with control cells transfected with vector plasmid, axons grew symmetrically from the explant. In contrast, when slit-myc transfected cells were used, axon growth was asymmetrical with more axons growing on the side of the explant distal to the slit expressing cells than on the side of the explant proximal to the slit expressing cells.

Example 9

Effect of Slit Protein on the Projection of

Olfactory Bulb Axons into the Telencephalon.

To confirm the effect of slit on axon guidance, the ability of slit to guide axon growth was assessed in whole-mount preparations of telencephalon, the natural environment for olfactory bulb axon growth.

Whole mount preparations of olfactory bulb-telencephalon co-culture were carried out with a protocol similar to that described in Sugisaki et al., *J. Neurobiol.* 29:127-137 (1996). Briefly, the telencephalic hemisphere together with the olfactory bulb were dissected out from mice at day 12.5 of gestation, freed from the pia mater, and placed on a collagen gel. Cells transfected with either vector alone or with *slit-myc* cDNA were labeled with DiO (3,3'dioctadecyloxacarbocyanine, Molecular Probes). Aggregates of these cells were put on top of the telencephalon, but not the olfactory bulb. Whole mount preparations were cultured with DMEM containing 10% fetal bovine serum at 37°C in 5% CO_2. Forty hours later, small crystals of the lipophilic dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine per-chlorate (DiI; Molecular Probes) were inserted into the olfactory bulbs. Eight to twelve hours later, the specimens were fixed with 4% paraformaldehyde in PBS and kept at 4 ^{0}C before examination under a fluorescent microscope.

The results obtained showed that while axons could grow into the part of a telencephalon covered with control, vector transfected cells, axons turned away from the part of a telencephalon covered with slit-myc transfected cells.

Example 10

Effect of Slit Protein on Neuronal Migration

Co-culture of subventricular zone (SVZa) explants from rats and slit expressing HEK cells was carried out in collagen gel matrices as previously described. Brains from newborn Sprague-Dawley (SD) rats (postnatal days 3-7) were embedded in 7% low melting point agarose prepared in phosphate buffered saline. Coronal and sagittal sections of 400 μm were cut with a vibratome. Tissue within the borders of the SVZ in coronal sections was dissected out to make SVZa explants of 200-400 μm in diameter. Septal explants were isolated form sagittal sections. Explants were placed into the collagen gel or matrigel as described previously (Hu and Rutishauser, *Neuron* 16:933-940 (1996); Wichterle et al., *Neuron*, 18:779-791 (1997)). The explants were cultured for 16 to 24 hours under 5% CO_2 in F-12

medium (Life Technologies) supplemented with 10% fetal bovine serum, penicillin and streptomycin. Aggregates of control or slit-expressing cells were made by the hanging drop method and placed into collagen gels or the matrigel together with SVZa explants and cultured as described above. For quantification, immunofluorescence images of sample stained with TuJ1 were obtained with a Zeiss microscope. Cell distribution was quantified by analyzing the distance between the cell body and the nearest edge of the explant.

As shown in Figure 4, when SVZa explants were co-cultured with HEK cells stably transfected with control plasmid, migrating cells symmetrically distributed around the circumference of the explants. When SVZa explants were co-cultured with cells stably transfected with a plasmid expressing the slit protein of the present invention, cell migration was highly asymmetric around each explant with more cells in the quadrant distal to the slit secreting cells than in the quadrant proximal to the slit cells. When the experiments were repeated using matrigel which allows migration of chains of cells, similar results were obtained with symmetrical chain migration around explants when co-cultured with control plasmid transfected HEK cells and asymmetrical chain migration when co-cultured with cell aggregates expressing the slit protein. The neuronal nature of the migrating cells was confirmed by staining with the TuJ1 antibody. When explants were cultured on top of slit expressing HEK cells, neurons still migrated out of the SVZa. These results show that slit is repulsive to migrating neurons.

To test whether slit can repulse neurons migrating in their natural pathway, brains were isolated from postnatal rats and sagittal sections cut with a vibratome. Slices of sagittal sections containing the SVZa, the rostral migratory stream (RMS) and the olfactory bulb (OB) were cultured in collagen gel matrices. Crystals of the lipophilic dye 1,1'-dioctadecyl-3, 3, 3', 3'-tetramethylindo-carbocyanine per chlorate (DiI) were inserted into the SVZa to label neuronal precursors and the slices were cultured as described for the SVZa explants. After 24 hours of culture, migrating neurons were found in the RMS. To test the effect of slit, control HEK cells or cells stably expressing slit were pre-labeled with 3,3' dioctacedyloxacarbocyanine (DiO), thus allowing visualization of SVZa neurons and HEK cells. DiI and DiO signals were visualized under a microscope using appropriate filter sets. Images were collected by photography using a Spot-camera (Zeiss) and analyzed.

When aggregates of control cells were placed on top of the RMS, cells from the SVZa migrated into the RMS. In contrast, when slit expressing cells were placed on the RMS, few cells migrated into the RMS (Figure 5). These results show that slit can regulate the migration of neuronal cells along their natural pathways.

The effect of slit on neuronal migration was also tested in GABAergic neurons of lateral ganglionic eminence (LGE) explants. As shown in Figure 6, when LGE explants were cocultured with control HEK cells transfected with the vector plasmid or with a plasmid expressing another axon repellent protein Semaphorin III, migrating neurons were symmetrically distributed around the circumference of the explants (Figure 6A). When LGE explants were co-cultured with HEK cells stably transfected with a plasmid expressing the slit protein of the present invention, cell migration was highly asymmetric around LGE explants with more cells in the quadrant distal to the slit expressing cell aggregates than in the quadrant proximal to the slit expressing cells (Figure 6C and Figure 6D). Human slit-2 and mouse slit-1 expressing cells have similar activity to the cells expressing Xenopus *slit* gene in these assays. These results indicated that slit proteins were chemorepellents to cells migrating out of LGE explants.

To test whether slit can act on LGE neurons in their natural migratory pathway, the striatal-cortical pathway, to direct neuronal migration from the LGE to the neocortex, a slice assay was used in which a coronal section of the rat embryonic brain containing the entire migratory pathway of LGE neurons was preserved in culture. Previous studies have established that DiI labeling in this system can trace GABAergic neurons migrating from the LGE to the neocortex (Anderson et al., Science 278, 474–476 (1997) ; Tamamaki et al., . J. Neurosci. 17, 8313–8323, (1997)). In this assay, slit-expressing cells or control cells were placed at the juncture of the LGE and the neocortex and examined their effects on cells migrating from the LGE to the neocortex (Figure 7). Slices of coronal sections of rat brains were labeled with Hoechst dye to reveal the outlines of the sections (Figure 7A and Figure 7E). Aggregates of Slit-expressing cells or control cells were labeled with 3, 3'dioctadecyloxacarbocyanine (DiO). In some experiments, slit or control cells were placed at the junction of the LGE and the neocortex (Figure 7B and Figure 7F, green cells). The migrating LGE neurons were traced by inserting a crystal of DiI in the subventricular zone of the LGE (Figure 7C and Figure 7G, red cells). Superimposition of three color images of Hoechst dye, DiO, and DiI revealed the positions of LGE cells migrating into the neocortex relative to the aggregates of Slit or control cells (Figure 7D and Figure 7H). In one

experiment with 53 explants, Slit-expressing cell aggregates were placed on the cortical-striatal junction on the left side of the brain and control cell aggregates were placed on the right junction. Among these explants, neurons migrated from the LGE past the region overlaid with an aggregate of control cells into the neocortex in 52 out of 53 slices. In 50 out of the same 53 slices but on the side with a Slit aggregate, no LGE neurons migrated past the region overlaid with Slit aggregates into the neocortex. In the three explants in which LGE neurons migrated past Slit cells, the migration of LGE neurons into the neocortex was significantly reduced by Slit aggregates when compared to control aggregates. These results indicate that Slit is a repellent for LGE neurons migrating in their normal pathway to the neocortex.

To test whether endogenous Slit contributes to the repulsive activity in the ventricular zone of the LGE, cells expressing RoboN, the extracellular fragment of the slit receptor roundabout (Robo), were used. RoboN can bind slit (Wu et al., *Nature* 400:331-336, (1999)) but cannot transduce the signal to intracellular compartments and is thus a competitive blocker of Slit. Aggregates of HEK 293T cells transfected with the control vector or with a plasmid expressing RoboN were made and placed at the bottom layer of the collagen gel matrix. Explants from the ventricular zone and those from the subventricular zone of the LGE were cocultured at the top layer. LGE neurons were repelled by the ventricular zone in the presence of control HEK 297T cells, whereas the presence of RoboN-expressing cells inhibited the repulsive activity of the ventricular zone. These results indicate that endogenous Slit present in the ventricular zone of the LGE is repulsive to LGE neurons.

The slit expressing cells were also tested in migration of cerebellar neurons. Rat cerebellar explants of 100-300mM were prepared with a vibratome and co-cultured either with the control HEK cells or cells expressing the slit protein. Migrating neurons were symmetrically distributed around the circumference of the explants when cultured with the control cells. When the cerebellar explants were co-cultured with HEK cells stably transfected with a plasmid expressing the slit protein of the present invention, cell migration was highly asymmetric around the cerebellar explants with more cells in the quadrant distal to the slit expressing cell aggregates than in the quadrant proximal to the slit expressing cells.

In several systems tested so far, including SVZa, LGE and cerebellar neurons, the human slit-2 protein has similar effect to the Xenopus slit protein. In addition, different fragments of slit protein were tested. Cells expressing amino-terminal fragments of Slit, including N890 (amino-terminal 890 amino acid residues) and N1076 (amino-terminal 1076

amino acid residues) behave similar to cells expressing full-length slit in repelling olfactory bulb axon. Slit fragment N890, however, did not have the repulsive activity in neuronal migration assay. Both full-length and slit fragment N1076 were active in repelling neuron in migration assay. The cells expressing the carboxyl terminal fragment C1 (nucleotide sequence encoding carboxyl-terminal 892-1529 amino acid residues fused to the sequence encoding the predicted signal peptide) did not show detectable activity in either axon guidance or neuronal migration assays.

Example 11

Effect of Slit on Cytokine Stimulated Cell Migration

The effect of slit on cell migration in response to cytokines was measured using the trans-well assay and the micro-chemotaxis chamber assay using either primary leukocytes or transfected HEK cells. Primary leukocytes were isolated from rat lymph node or peripheral blood using standard methods (Coligan et al., eds., *Current Protocols in Immunology*, Wiley & Sons, (1991)). HEK cells were co-transfected by the calcium phosphate method or by electroporation with plasmids encoding the slit protein receptor roundabout and the chemokine receptor CXCR4 (Robo + CXCR4) or co-transfected with plasmids encoding roundabout and the chemokine receptor CCR5 (Robo+CCR5). CXCR4 receptors bind SDF-1 and CCR5 receptors bind RANTES (Baggiolini et al., *Annu. Rev. Immunol.*, 15:675-705, 1997). Leukocytes or transfected HEK cells were kept on ice in DMEM supplemented with 5-10% heat inactivated fetal bovine serum. Immediately before being transferred into the chemotaxis chamber, cells were resuspended at 1-5 x 10^6 cells/ml in either DMEM, RPMI or 50% RPMI + 50% M199 medium (Life Technologies) without serum, but containing 0.25-0.5% BSA.

The chemotaxis assays were preformed as previously described (Taub et al., *J. Immunol. Meth.*, 184:1870198 (1995); Ganju et al., *J. Biol. Chem.*, 273:23169-23175 (1998)). Brielfy, media were put in the bottom wells containing different concentrations of commercially available recombinant RANTES or SDF1 (R&D Systems) or test proteins (slit and its derivatives) or combinations of cytokine and purified slit proteins. Approximately 2-5 x 10^4 cells were placed in the top wells of a 48 well micro-chemotaxis chamber (NeuroProbe) or 1-5 x 10^5 cells in the inner inserts of a trans-well chamber (Costar). The upper and lower wells were separated by either 5 μm polycarbonate filters for leukocytes or 8 μm polycarbonate filters for transfected HEK cells. Cells were incubated at 37°C for 80-240

minutes. Cells that migrated into the bottom chamber were counted under a microscope with a hemacytometer. Cells that migrated into the pores of the filter were counted after fixation in methanol and staining in Diff-Quick solution (Baxter).

In one experiment, the effect of differing concentrations of the cytokines RANTES and SDF on leukocyte migration in the presence of 0, 100 pM or 200 pM purified slit protein was determined. Concentrations of RANTES ranged from 10^{-6} M to 10^{-12} M with control wells containing no RANTES. Concentrations of SDF ranged from 10^{-6} M to 10^{-12} M. The results of the experiment using RANTES are shown in Figure 8A. When no slit protein was present, increasing concentrations of RANTES resulted in increased migration of leukocytes up to 10^{-9} M at which point a plateau was reached. Addition of slit protein markedly decreased the migration, with migration completely inhibited at 200 pM slit. Similar results were seen with SDF (Figure 9A) except that leukocyte migration was completely inhibited with slit at either 100 or 200 pM. The reciprocal experiment was also conducted in which the concentration of purified slit varied between 0 and 1100 pM while the concentration of cytokine, RANTES or SDF, was held constant at 10^{-9} M. The results obtained were similar for either RANTES (Figure 8B) or SDF (Figure 9B). In both cases, addition of purified slit protein at a concentration of 200 pM completely abolished chemokine stimulated leukocyte migration.

In another set of experiments, the effect of purified slit on cytokine stimulated cell migration was examined using transfected HEK cells expressing robo, the CCR5 receptor, the CXCR4 receptor, robo+ CCR5, or robo+CXCR4. In one experiment the concentration of RANTES varied from 10^{-6} to 10^{-12} M, while the concentration of purified slit was either 0 or 100 pM. The results of this experiment are shown in Figure 10. Increasing concentration of RANTES up to 10^{-9} M resulted in increased cell migration in cells expressing CCR5 alone regardless of the presence of slit at 100 pM. Slit protein at 100 pM, however, completely inhibited migration of cells transfected with both Robo and CCR5. Similar results were observed when the experiment was repeated with SDF as the chemokine (Figure 11). As with RANTES, inhibition of cell migration by 100 pM slit was only observed in cells transfected with both Robo and CXCR4 receptor.

Purified human slit 2 protein has the activity similar to Xenopus slit in inhibiting leukocyte migration induced by RANTES and SDF1, as shown in Figure 12 and Figure 13. In addition, carboxyl terminal fragment C1 of slit showed similar activity in inhibiting leukocyte migration induced by RANTES and SDF1.

Example 12

Effect of Slit on Cell Proliferation

HEK cells were stably transformed as previously described with either slit or control vector. Cells were cultured in DMEM as previously described. After 2 and 5 days of culture the number of cells present was determined either by direct counting or by counting on a hemacytometer after staining with trypan blue. Cells that stably expressed slit were found to proliferate at 2-3 times the rate of vector transformed cells.

HEK cells stably transformed with either slit or control vector were cultured in DMEM as previously described except that the concentration of fetal bovine serum was reduced over a 2-3 week period from 10% to 2% in 2% increments. The reduction in serum concentration had no effect on proliferation of slit transformed cells. In contrast, vector transformed cells showed significant reductions in rate of proliferation as the serum concentration decreased.

Mesencephalic neuronal cells were cultured in medium that had been conditioned with either cells secreting the slit protein or cells secreting the lunatic fringe protein. When compared to cells cultured in medium conditioned with lunatic fringe, neuronal cells cultured in slit conditioned medium were more granular and had thicker neuritic masses indicating neurotrophic activity. These results indicate that slit has an affect on cell proliferation.

Conclusion

In light of the detailed description of the invention and the examples presented above, it can be appreciated that the several aspects of the invention are achieved.

It is to be understood that the present invention has been described in detail by way of illustration and example in order to acquaint others skilled in the art with the invention, its principles, and its practical application. Particular formulations and processes of the present invention are not limited to the descriptions of the specific embodiments presented, but rather the descriptions and examples should be viewed in terms of the claims that follow and their equivalents. While some of the examples and descriptions above include some conclusions about the way the invention may function, the inventors do not intend to be bound by those conclusions and functions, but put them forth only as possible explanations.

It is to be further understood that the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention, and that many alternatives, modifications, and variations will be apparent to those of ordinary skill in the art

in light of the foregoing examples and detailed description. Accordingly, this invention is intended to embrace all such alternatives, modifications, and variations that fall within the spirit and scope of the following claims.

What is claimed is

1. An isolated polynucleotide comprising a member selected from the group consisting of:
   (a) a polynucleotide of SEQ ID NO: 1 or the complement thereof;
   (b) a polynucleotide that has at least 90% sequence identity with the polynucleotide of (a);
   (c) a polynucleotide that hybridizes to the polynucleotide of (a) under conditions of 5X SSC, 50% formamide and 42°C, and which encodes a protein having the same biological function;
   (d) a polynucleotide encoding the same amino acid sequence as (a), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code;
   (e) a polynucleotide encoding the same amino acid sequence as (b), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; and
   (f) a polynucleotide encoding the same amino acid sequence as (c), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code.

2. A recombinant polynucleotide comprising a member selected from the group consisting of:
   (a) a polynucleotide of SEQ ID NO: 1 or the complement thereof;
   (b) a polynucleotide that has at least 90% sequence identity with the polynucleotide of (a);
   (c) a polynucleotide that hybridizes to the polynucleotide of (a) under conditions of 5X SSC, 50% fromamide and 42°C, and which encodes a protein having the same biological function;
   (d) a polynucleotide encoding the same amino acid sequence as (a), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code;
   (e) a polynucleotide encoding the same amino acid sequence as (b), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; and

(f) a polynucleotide encoding the same amino acid sequence as (c), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code.

3. A recombinant vector comprising a member selected from the group consisting of:

(a) a polynucleotide of SEQ ID NO: 1 or the complement thereof;

(b) a polynucleotide that has at least 90% sequence identity with the polynucleotide of (a);

(c) a polynucleotide that hybridizes to the polynucleotide of (a) under conditions of 5X SSC, 50% formamide and 42°C, and which encodes a protein having the same biological function;

(d) a polynucleotide encoding the same amino acid sequence as (a), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code;

(e) a polynucleotide encoding the same amino acid sequence as (b), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; and

(f) a polynucleotide encoding the same amino acid sequence as (c), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code.

4. The recombinant vector of claim 3 further comprising at least one addition sequence chosen from the group consisting of:

(a) regulatory sequences operatively coupled to the polynucleotide;

(b) selection markers operatively coupled to the polynucleotide;

(c) marker sequences operatively coupled to the polynucleotide;

(d) a purification moiety operatively coupled to the polynucleotide;

(e) a secretion sequence operatively coupled to the polynucleotide; and

(f) a targeting sequence operatively coupled to the polynucleotide.

5. The recombinant vector of claim 3 wherein said vector further comprises a developmentally-regulated, an organelle-specific, a tissue-specific, or cell-specific promoter.

6. A host cell comprising the recombinant vector of claim 3.

7. The host cell of claim 6, wherein said host cell is selected from the group consisting of mammalian cells, plant cells, insect cells, yeast, bacteria, bacteriophage.

8. The host cell of claim 6, wherein said host cell expresses a protein encoded by said vector.

9. The host cell of claim 8, wherein said expressed protein is secreted by said host cell.

10. A protein or polypeptide fragment encoded by the polynucleotide selected from the group consisting of:

(a) a polynucleotide of SEQ ID NO: 1 or the complement thereof;

(b) a polynucleotide that has at least 90% sequence identity with the polynucleotide of (a);

(c) a polynucleotide that hybridizes to the polynucleotide of (a) under conditions of 5X SSC, 50% formamide and 42°C, and which encodes a protein having the same biological function;

(d) a polynucleotide encoding the same amino acid sequence as (a), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code;

(e) a polynucleotide encoding the same amino acid sequence as (b), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; and

(f) a polynucleotide encoding the same amino acid sequence as (c), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code.

11. A protein comprising the amino acid sequence of SEQ ID NO: 2 or a fragment of SEQ ID NO: 2.

12. The protein of claim 10, wherein one or more of the amino acids have been substituted with a conserved amino acid and the biological function of the protein has been maintained.

13. The protein of claim 10, wherein one of more of the amino acid resides includes a substituent group.

14. A method for the production of a purified slit protein comprising growing host cells of claim 8 under conditions where said host cells express a protein encoded by said recombinant vector and isolating said expressed protein.

15. A pharmaceutical composition comprising the protein of claim 10, a protein fragment thereof, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier, diluent, or excipient.

16. A method for altering cell migration comprising administering a migration altering amount of a slit protein or a pharmaceutically acceptable salt thereof.

17. The method of claim 16 wherein said slit protein is the protein of claim 10.

18. The method of claim 16, wherein said alteration is by repulsion.

19. The method of claim 16, wherein said alteration is by inhibition.

20. The method of claim 16, wherein said cells express the roundabout (robo) protein.

21. The method of claim 16, wherein said cells are neural cells.

22. The method of claim 16, wherein said cells are malignant cells.

23. The method of claim 16, wherein said cells are leukocytes.

24. The method of claim 16, wherein said migration inhibiting amount is in the range of from about 5 pM to about 1 mM.

25. The method of claim 16, wherein said protein is administered locally.

26. The method of claim 25, wherein said protein is administered by transplantation of transformed cells secreting said protein.

27. The method of claim 25, wherein said protein is administered by a transplantable mini pump.

28. A method for guiding cell migration comprising administering an effective amount of a slit protein or a pharmaceutically acceptable salt thereof.

29. The method of claim 28, wherein said protein is the protein of claim 10.

30. The method of claim 28, wherein said effective amount is in the range of from about 5 pM to about 1mM.

31. A method for guiding nerve axons or dendrites comprising administering a dendrite or axon guiding amount of a slit protein.

32. The method of claim 31, wherein said protein is the protein of claim 10.

33. The method of claim 31, wherein said effective amount is in the range of from about 5 pM to about 1 mM.

34. A method for the treatment of graft rejection comprising administering a leukocyte migration inhibiting amount of a slit protein or a pharmaceutically acceptable salt thereof.

35. The method of claim 34, wherein said protein is the protein of claim 10.

36. The method of claim 35, wherein said migration inhibiting amount is in the range of from about 5 pM to about 1 mM.

37. The method of claim 34, wherein said protein is administered locally.

38. The method of claim 37, wherein said protein is administered by transplantation of transformed cells secreting said protein.

39. The method of claim 37, wherein said protein is administered by a transplantable mini pump.

40. A method for increasing cell proliferation comprising transforming cells with the vector of claim 3.

41. A method for culturing cells comprising the addition of a slit protein to the culture medium.

42. The method of claim 41 wherein said slit protein is the protein of claim 10.

43. A cell culture medium comprising a slit protein or a slit protein derivative.

44. The cell culture medium of claim 43, wherein said slit protein is the protein of claim 10.

45. The cell culture medium of claim 43, wherein said culture medium is a reduced serum medium.

46. The cell culture medium of claim 43, wherein said culture medium is a serum-free culture medium.

47. A composition comprising the protein of claim 10.

48. The composition of claim 47, wherein said protein is added to cell culture medium in place of serum.

49. An expression cassette comprising the polynucleotide of claim 1, a promoter, and a transcriptional termination signal sequence.

50. A method for inhibiting the infection of a cell by HIV comprising administering an HIV infection inhibiting amount of the slit protein.

51. The method of claim 50, wherein said slit protein is the slit protein of claim 10.

52. The method of claim 50, wherein the infection inhibiting amount of said protein is in the range of from about 5 pM to about 1 mM.

53. A method for aiding wound repair comprising administering a cell proliferation stimulation amount of slit protein, a slit protein derivative, or mixtures thereof.

54. The method of claim 53, wherein said slit protein is the protein of claim 10.

55. A method for aiding in organ regeneration comprising administering a cell proliferation stimulating amount of a slit protein, a slit protein derivative, or mixtures thereof.

56. The method of claim 55, wherein said slit protein is the protein of claim 10.

57. A method for inhibiting inflammation comprising administering a leukocyte migration inhibiting amount of a slit protein, a slit protein derivative, or mixtures thereof.

58. The method of claim 57, wherein said slit protein is the protein of claim 10.

FIGURE 1A

GAATTCGGCACGAGAGCAGCATGGGAACTGTATTCCCTGTATTTACACAAGGCTCAACTGAGATGCTATGCAGTG
ACTCCAACTAAGTTCTGCTGAATATACCTGCCTGGTGGGCTGACATTTCCTGCATAGACTAAGCCTAAGGATATT
CGAGCCATACAAAAGCCACACAGGGATTCTGACGGAGATACAGAGAAAGAAAGAAAAAAGAAAATCAGTCACTGCC
TGAAGGATTGCTGGGGCTGCTGTAGGCACAAAATCACAACCATCAACTCTGTGCGCTCTGACAGGAATATAAAAG
CTTTGCTCCCAGCCACGACAAATTGCCTTTGGATTTTGGAGGTGTGCACCAAGCATGCATTGTGGATAAACAAGT
GGGGTGCTTCCTATTGCACAAACTTGTGAGTTAAGAAAAAGAAGAAAAAAAGAAAAAAAAATCCCCCTACAAGAT
CAGATTTGGGTGGCAGTTGTCCTGGGTTTGGTGACATCTAACAGTTTTTGGTTGGGTTCTTTCCTTGGGTGGCTT
TGTGTTTTTTTTTCATTTTTGACCAAATGTTACCAAGTTTTGTGAAGAAGAAGATGTGCTATATTGGTTTCTGGAA
GCTGTCCATCTCTTTGGGTTTGGTACTGGTCATACTCAGCGAGGTGGCACCTCAGCCTTGTCCATCTCAGTGCTC
TTGCACAGGCACCACCGTGGACTGCCATGGGCTCTCCCTACGTAGCGTGCCACGCAACATCCCAAGAAACGCCGA
GCGCCTAGACCTGAATGCCAATAACATCACTAGAATCTCCAAAACAGACTTTGCTGGATTAAGACACCTCCGGAT
CTTGCAGCTTATGGAGAACAAGATTACTACAATTGAGAGGGGAGCATTCCAAGACCTGAAAGAACTGGAAAGACT
GCGCCTTAACCGAAATAATCTGCAGGTGTTCCCTGAACTGCTTTTCCTGGGGACTCCAAAACTCTACAGACTTGA
CCTCAGTGAGAACCAGATCCAAGCCATCCCAAGAAAGGCATTCCGTGGTGCAGTAGATATTAAAAACCTGCAGCT
GGATTATAACCAGATCAGCTGTATTGAAGATGGGACATTCCGAGCACTGCGTGACCTGGAAGTACTCACTCTCAA
CAACAATAACATTACTCGACTTTCGGTGGCAAGCTTCAATCACATGCCGAAACTCAGGACCTTTCGACTCCATTC
CAACAACTTGTACTGTGATTGTCACCTCGCTTGGTTATCCGACTGGCTGCGTCAGAGGCCCCGAGTTGGTCTCTA
CACTCAGTGTATGGGACCCATTCACCTGAGAGGGCATAATGTTGCTGAGGTTCAGAAGCGAGAATTCAGCTGCAC
AGGTCAACAGTCATTTATGGTCCATTCTTGTAGTGTCTTGCATTGCCCTGCTGCTTGTACCTGCAGTAACAATAT
TGTAGATTGCCGTGGAAAAGGGTTGACTGAGATCCCAACCAACCTCCCGGAGACAATAACAGAAATGAGATTGGA
GCAAAATTCGATCAAGGTCATTCCTCCAGGAGCATTCTCACCATACAAGAAGTTGAGAAGAATTGATTTAAGCAA
CAACCAGATTTCTGAAATAGCCGCAGATGCTTTCCAAGGCCTGCGTTCACTCAACTCCCTTGTTCTCTATGGAAA
TAAAATTACAGAGCTCCCCAAAGGCTTGTTTGAAGGACTGTTCTCATTACAGTTACTATTACTCAATGCCAACAA
GATTAATTGTTTACGAGTAGACTCCTTCCAAGATCTGCACAACCTGAACCTCCTATCTTTGTATGACAACAAACT
TCAAACCATCGCCAAAGGCACATTCTCCCCACTCCGTGCCATCCAGACATTGCATTTGGCTCAGAACCCGTTTAT
CTGTGACTGCCATCTGAAGTGGCTGGCGGATTATCTCCATACAAACCCTATTGAGACCAGTGGTGCCCGCTGCAC
CAGCCCCCGCCGACTGGCAAACAAAAGGATTGGGCAGATCAAAAGCAAGAAATTCCGCTGCTCAGGCACAGAAGA
CTACCGTTCCAAGCTTAGTGGAGACTGTTTTGCAGACTTGGCCTGTCCAGAAAAATGCCGGTGTGAAGGAACCAC
TGTGGACTGTTCCAACCAAAAACTCACAAAGATCCCTGACCATATTCCCCAGTACACAGCTGAGTTGCGCCTTAA
CAACAATGAATTCACAGTTTTGGAGGCAACTGGAATCTTCAAGAAACTTCCCCAGCTAAGAAAAATCAATCTGAG
CAACAACAAGATAACAGATATAGAAGAAGGGGCTTTCGAGGGGGCTAATGGCGTGAATGAGCTATTGTTAACAAG
TAACCGTATGGAAAATGTTCGCCATAAGATGTTCAAGGGCCTGGAGGGACTGAAAACCCTGATGCTCAGAAGCAA
TCACATTAGTTGTGTGAATAATGACAGCTTCACAGGACTGAGCTCAGTTCGCCTGCTCTCCCTCTATGACAATCA
AATTACTACAGTGGCTCCAGGTGCATTCGACACACTACATTCTCTCTCTACCTTGAACCTTTTAGCAAATCCATT
CAACTGCAACTGCCACCTGGCTTGGTTGGGTGACTGGCTTAGGAAGAAGCGCATTGTCACTGGAAACCCTCGATG
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TCTTTCTAACCACAGCTTCAGCAACATGACTCACCTCCTTACCTTGATTCTTAGTTACAATCGTCTTAGGTGCAT
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TTTACCAAAACAATCATTACTAAGCGTTGATTCCACACTTTACATAGGAGGTATGCCCATGAAAAACAACATTGC
CGGTCTTCGACAGTCTTCAAGTCAAAATGGTACCAACTTCCAAGGTTGCATTAGAAACCTATACATTAATGATGA

Figure 1B

ACTACAGGACTTCAGAAAACTAAACTTACAACCTGGAGTTCTTCCCGGCTGTGAACCATGCCATAAAAATGTCTG
CGTCCATGGCACATGCCTGGCTACAAGCCAAGCCGGCTTCACTTGCGAATGTGAAGGAGGATGGACAGGGCAACT
CTGCGATATCCAGTCCAATGATCCCTGTCTGGGAAACAAGTGTGTACATGGCACATGCCTCCCAATTAATTCATT
CTCCTACAGTTGTAAATGCCTTCAAGATTTTGTGGGAGTTCTCTGTGATGAAGCTGAAGATCAACCCAATCCCTG
CCAGTCCATTAAATGTAAACATGGAAAGTGCAGGCTTTCAGGCTTAGGAAAAGCATACTGTGAATGCTCCAGTGG
TTATACTGGAGACAACTGTGATAAAGAAATCTCTTGCCGAGGGGAACGGATCAGAGATTACTACCAAAGGCAGCA
AGGTTATGCTGCTTGCCAGACGACCGAGAAGGTATCGAGACTAGAATGCAAAGGAGGATGCTCAAATGGTCAGTG
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TGAGAAAATCATAAAGTGTGGTTGTACAAAGTGCCCCTCCTAAACATGTAAACACCACAATTCTTTTGAAAATGT
TGTATACCTATTGCCCATGTTGGACTAATTAAAGTTTCATAGTGGAAATATTTGAATTATATTGTAAAATACAGA
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AAAGAGAATGAGTTGCTGATGCCTGAACTTTGAATATGAGGAGCTGAAATAGGAACTAAAAAAATGTTGTATGGA
GATTCAGCTTCATTGAGGGGTAATGCAATTTCTTTCAGAAACAGTCACATACTGAAGATACAATTAGAAATCATT
TGGCCAGGACAATGAAAAAAAAAAAAAAAAAACTCGAG

FIGURE 2

MLPSFVKKKMCYIGFWKLSISLGLVLVILSEVAPQPCPSQCSCTGTTVDCHGLSLR
SVPRNIPRNAERLDLNANNITRISKTDFAGLRHLRILQLMENKITTIERGAFQDLK
ELERLRLNRNNLQVFPELLFLGTPKLYRLDLSENQIQAIPRKAFRGAVDIKNLQLD
YNQISCIEDGTFRALRDLEVLTLNNNNITRLSVASFNHMPKLRTFRLHSNNLYCDC
HLAWLSDWLRQRPRVGLYTQCMGPIHLRGHNVAEVQKREFSCTGQQSFMVHSCSVL
HCPAACTCSNNIVDCRGKGLTEIPTNLPETITEMRLEQNSIKVIPPGAFSPYKKLR
RIDLSNNQISEIAADAFQGLRSLNSLVLYGNKITELPKGLFEGLFSLQLLLLNANK
INCLRVDSFQDLHNLNLLSLYDNKLQTIAKGTFSPLRAIQTLHLAQNPFICDCHLK
WLADYLHTNPIETSGARCTSPRRLANKRIGQIKSKKFRCSGTEDYRSKLSGDCFAD
LACPEKCRCEGTTVDCSNQKLTKIPDHIPQYTAELRLNNNEFTVLEATGIFKKLPQ
LRKINLSNNKITDIEEGAFEGANGVNELLLTSNRMENVRHKMFKGLEGLKTLMLRS
NHISCVNNDSFTGLSSVRLLSLYDNQITTVAPGAFDTLHSLSTLNLLANPFNCNCH
LAWLGDWLRKKRIVTGNPRCQKPYFLKEIPIQDVAIQDFTCDDGNEDNSCSPLSRC
PAECTCLDTVVRCSNKGLKSLPKGIPKEVTELYLDGNVFPLVPKELSNYMHLTLID
LSNNQISTLSNHSFSNMTHLLTLILSYNRLRCIPLRAFDRLKSLKLLSLHGNDVSA
IPEGAFSDLSALSHLAIGANPLYCDCKMQWLSDWVKSEYKEPGIARCSGPGEMADK
LLLTTPSKKFSCNGPVDVNILAKCNPCLSNPCKNNGTCNNDPVEFYRCTCPYGFKG
QDCDVPIHACISNPCHNGGTCHLKEGDKEGFWCACTDGFEGENCEVNIDDCEDNDC
ENNSTCVDGINNYTCRCPPEYTGELCEEKLDFCSQDINPCQHDSKCIQTLKGYKCD
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PRTSPCDNFECQNGAQCIVKGNDLLCQCLPGYRGNKCEKLVSVNFVNKDSFIPIPS
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VETINDGNFHVVELVNLDQTLSLIIDGGTPKSITNLPKQSLLSVDSTLYIGGMPMK
NNIAGLRQSSSQNGTNFQGCIRNLYINDELQDFRKLNLQPGVLPGCEPCHKNVCVH
GTCLATSQAGFTCECEGGWTGQLCDIQSNDPCLGNKCVHGTCLPINSFSYSCKCLQ
DFVGVLCDEAEDQPNPCQSIKCKHGKCRLSGLGKAYCECSSGYTGDNCDKEISCRG
ERIRDYYQRQQGYAACQTTEKVSRLECKGGCSNGQCCGPLRSKRRKYAFECTDGSS
FVDEVEKIIKCGCTKCPS

*Sequence uses the standard single letter amino acid abbreviations.

FIGURE 3A

```
1    tgccccgcgc gctctgctac gggcccgctg ggcctccgcg ccttctagct tccggagccc
61   actttgatcg gggccataat acctattgag atcccctctt ctgtcttgta ccttcgccac
121  tggcatcgga tttgcagaag cctgcgtggg atcagaggac cgccctcccc acaacaaccg
181  gcccctgcat cttagcagcc gttggaagcc ccagctcttt taccgccaag ttcatccttg
241  ggagacagaa gacgcgtgat ctcctctccg ctgctcttgg ggtctccttg cagccctggc
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421  ttttgtgcac attttccctg gcactctggg ttgctagccc cgccgggcac tgggcctcag
481  acactgcgcg gttccctcgg agcagcaagc taaagaaagc ccccagtgcc ggcgaggaag
541  gaggcggcgg ggaaagatgc gcggcgttgg ctggcagatg ctgtccctgt cgctggggtt
601  agtgctggcg atcctgaaca aggtggcacc gcaggcgtgc ccggcgcagt gctcttgctc
661  gggcagcaca gtggactgtc acgggctggc gctgcgcagc gtgcccagga atatcccccg
721  caacaccgag agactggatt taaatggaaa taacatcaca agaattacga agacagattt
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841  aagaggagca ttccaggatc ttaaagaact agagagactg cgtttaaaca gaaatcacct
901  tcagctgttt cctgagttgc tgtttcttgg gactgcgaag ctatacaggc ttgatctcag
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1021 tttgcaactg gattacaacc agatcagctg tattgaagat ggggcattca gggctctccg
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1441 cacaaatctt ccagagacca tcacagaaat acgtttggaa cagaacacaa tcaaagtcat
1501 ccctcctgga gctttctcac catataaaaa gcttagacga attgacctga gcaataatca
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1741 ccacaacttg aaccttctct ccctatatga caacaagctt cagaccatcg ccaaggggac
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2221 aaactttagc aacaataaga tcacagatat tgaggaggga gcatttgaag gagcatctgg
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3601 ttgcccacct gagtatacag gtgagttgtg tgaggagaag ctggacttct gtgcccagga
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Figure 3B

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3841 cgaaggttac agtggcttgt tctgtgagtt ttctccaccc atggtcctcc ctcgtaccag
3901 cccctgtgat aattttgatt gtcagaatgg agctcagtgt atcgtcagaa taaatgagcc
3961 aatatgtcag tgtttgcctg gctatcaggg agaaaagtgt gaaaaattgg ttagtgtgaa
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4201 tcatccagct tctgccattt acagtgtgga gacaatcaat gatggaaact tccacattgt
4261 ggaactactt gccttggatc agagtctctc tttgtccgtg gatggtggga accccaaaat
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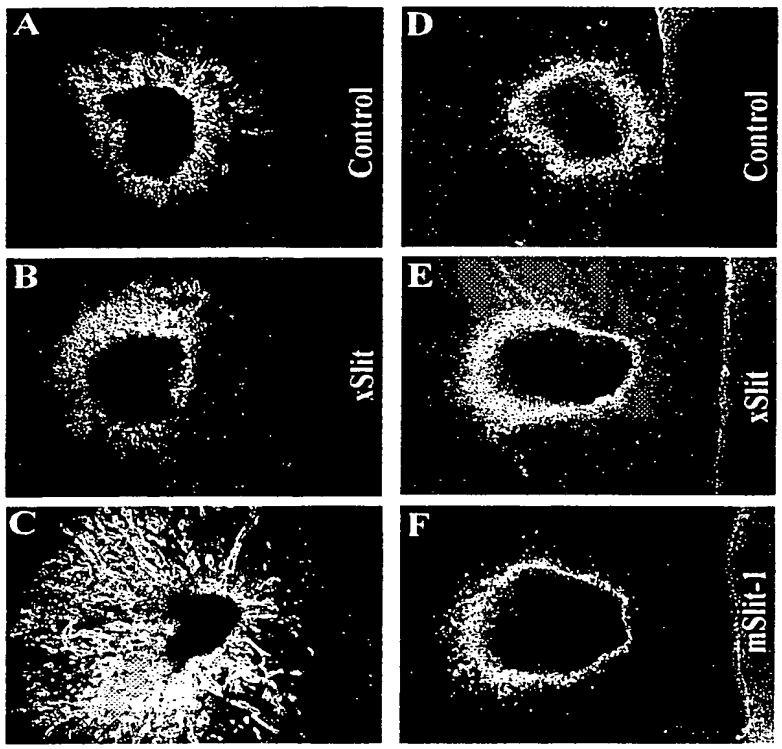
FIGURE 4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D
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B
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E
xSlit
C
F
mSlit-1
Matrigel
Collagen Gel

FIGURE 5

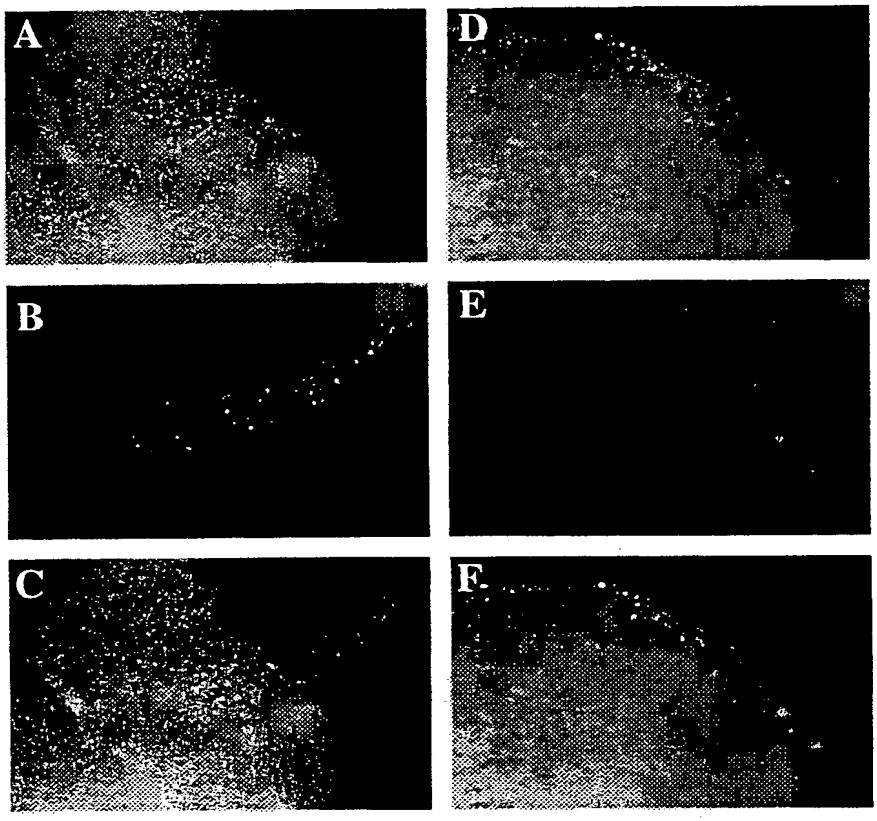
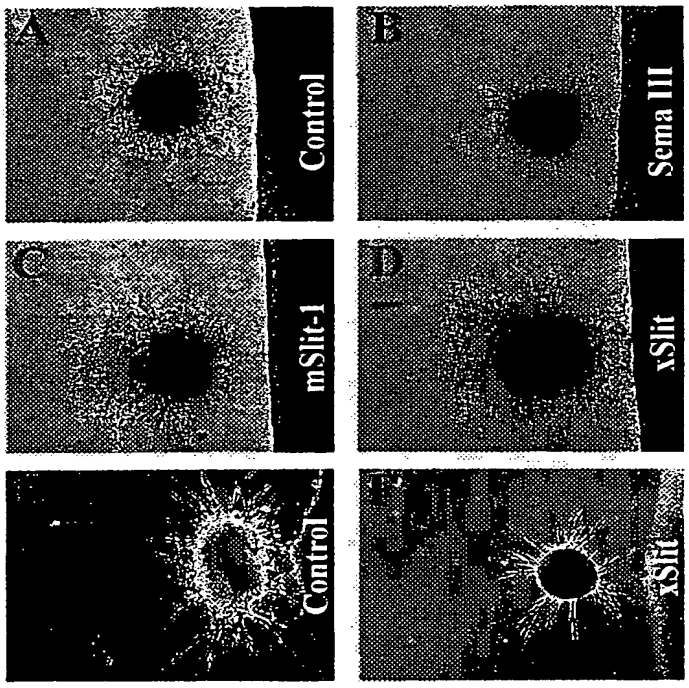


FIGURE 6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B
Sema III
C
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D
xSlit
E
Control
F
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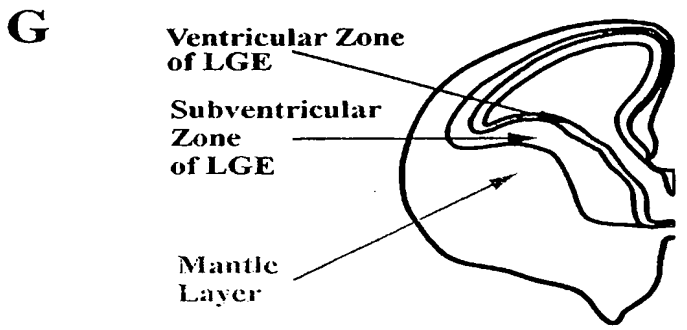
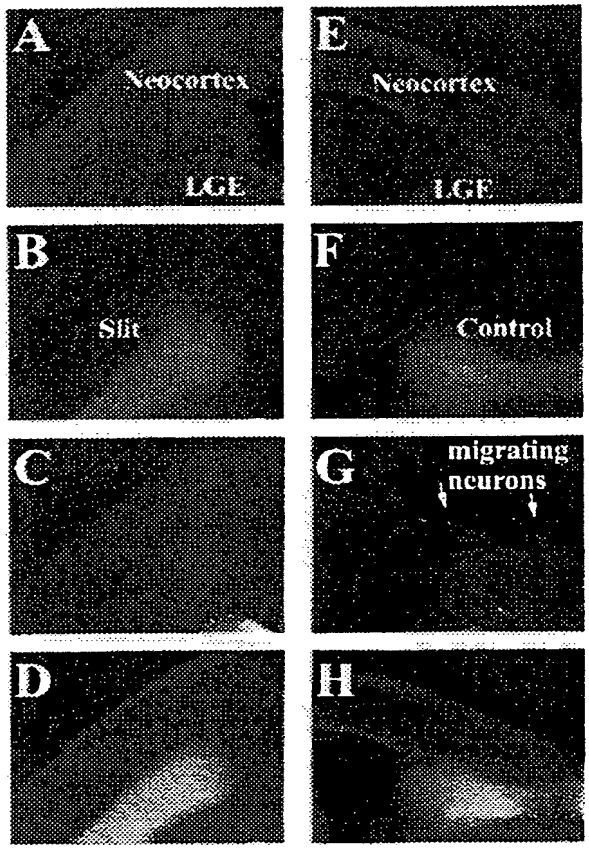

G
Ventricular Zone of LGE
Subventricular Zone of LGE
Mantle Layer

FIGURE 7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Neocortex
LGE
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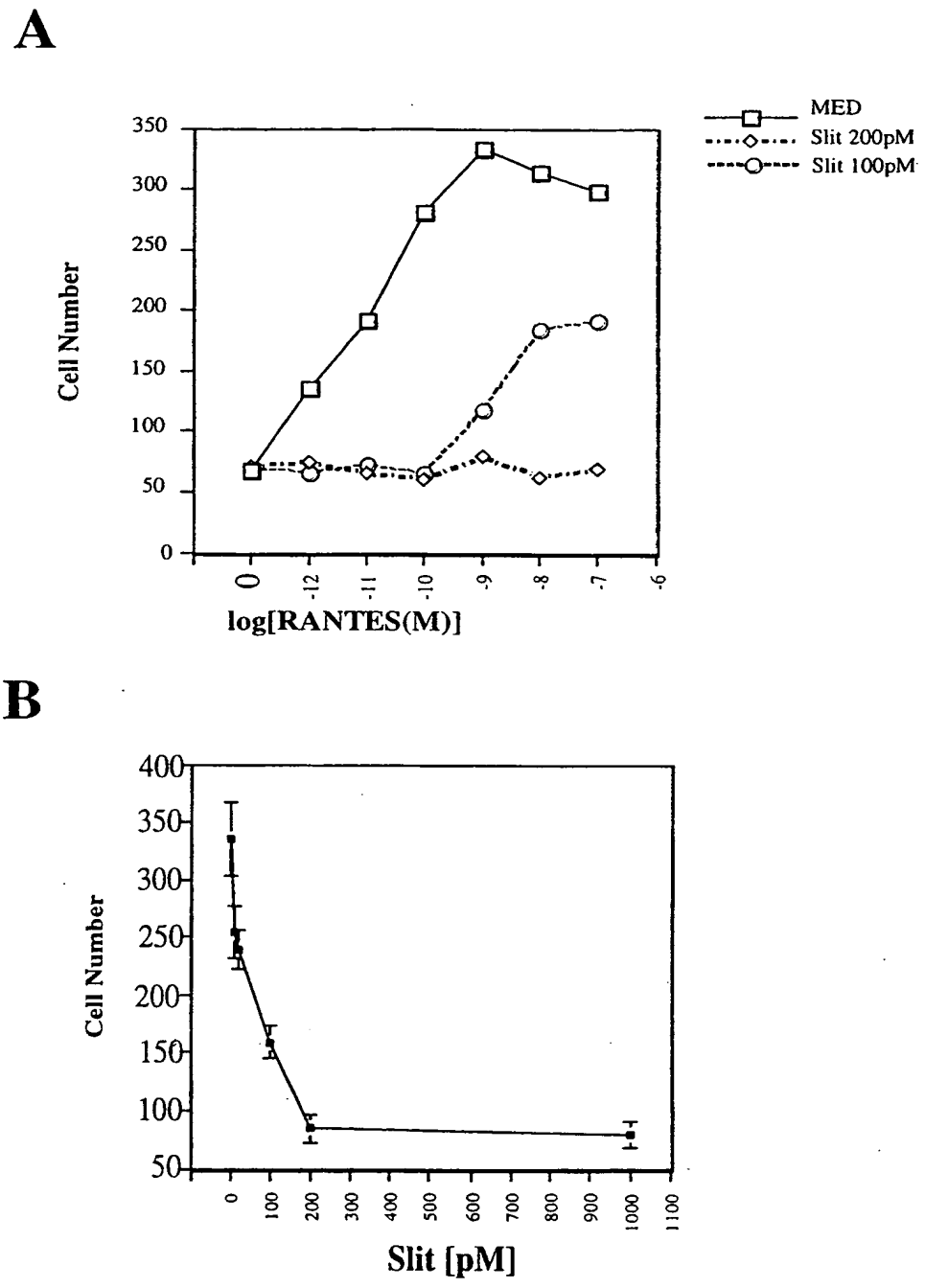
Neocortex
LGE
B
Slit
F
Control
C
G
migrating
neurons
D
H

Fig. 8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Slit 100pM
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-10
-9
-8
-7
-6
log[RANTES(M)]
B
Cell Number
400
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300
250
200
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700
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900
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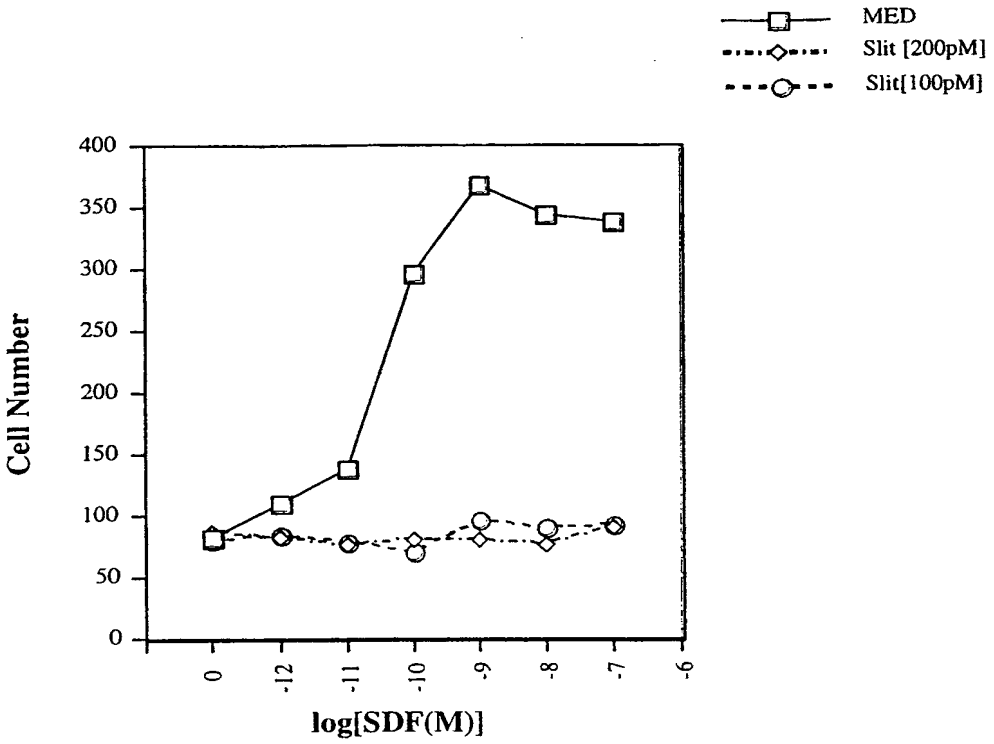
Slit [pM]

Fig. 9

A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-11
-10
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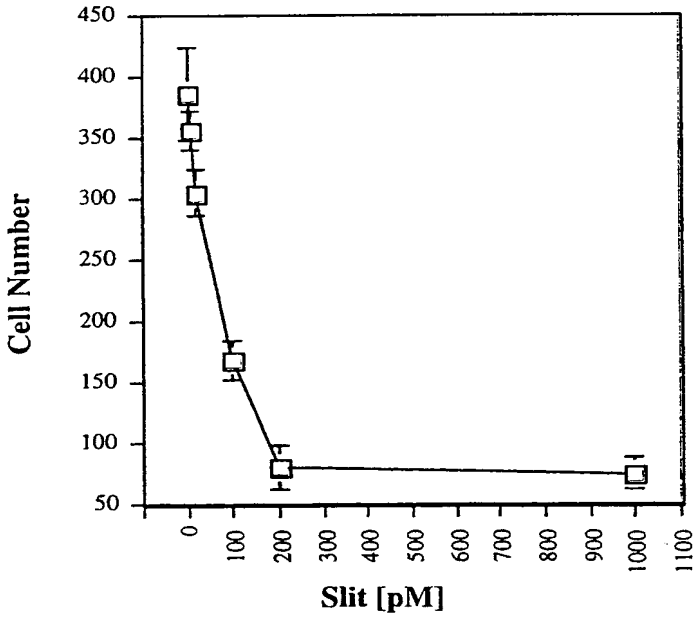
-8
-7
-6
log[SDF(M)]

B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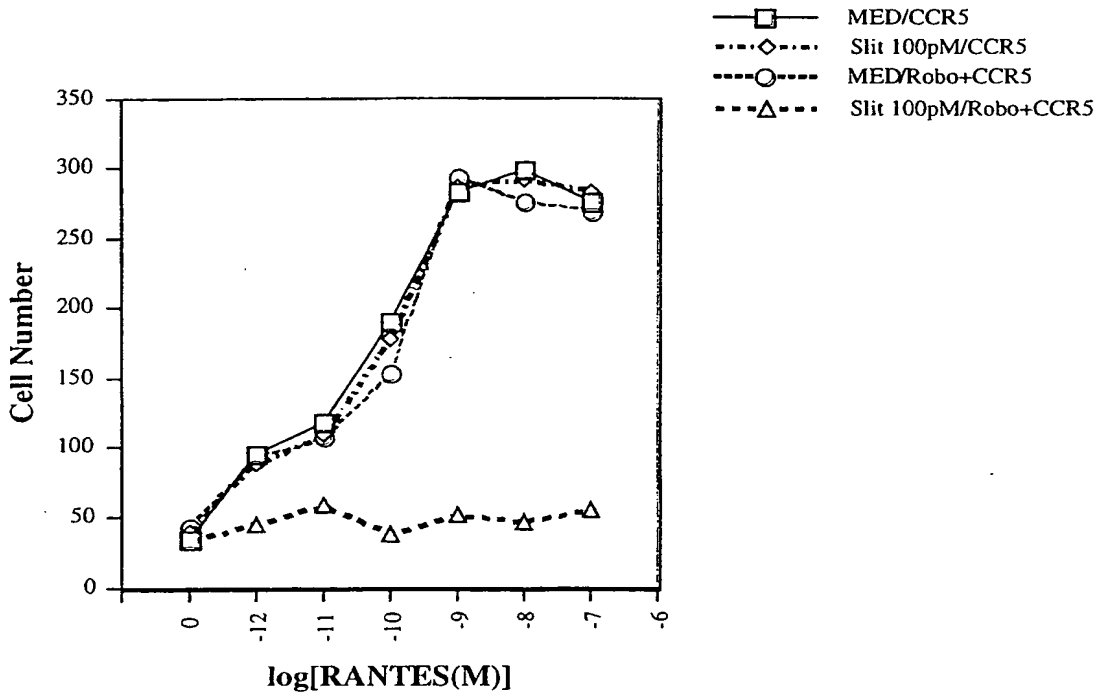
1100
Slit [pM]

Fig. 10


MED/CCR5
Slit 100pM/CCR5
MED/Robo+CCR5
Slit 100pM/Robo+CCR5
Cell Number
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300
250
200
150
100
50
0
0
-12
-11
-10
-9
-8
-7
-6
log[RANTES(M)]

Fig. 11

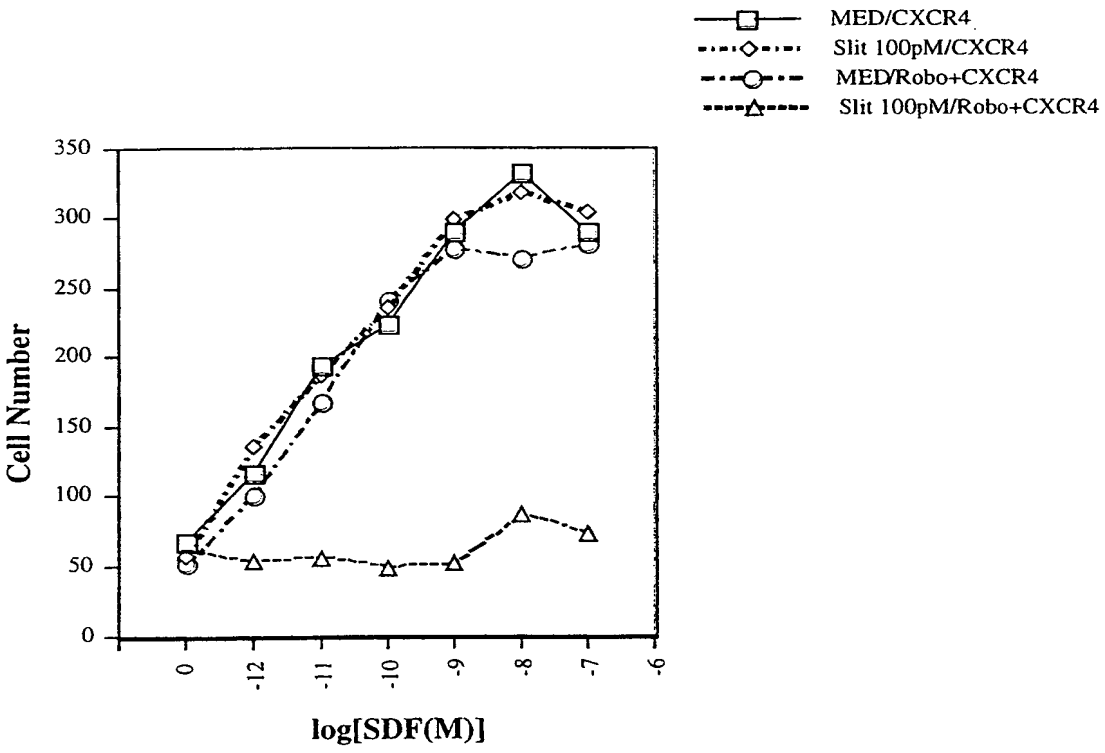

MED/CXCR4
Slit 100pM/CXCR4
MED/Robo+CXCR4
Slit 100pM/Robo+CXCR4
Cell Number
350
300
250
200
150
100
50
0
0
-12
-11
-10
-9
-8
-7
-6
log[SDF(M)]

Fig. 12

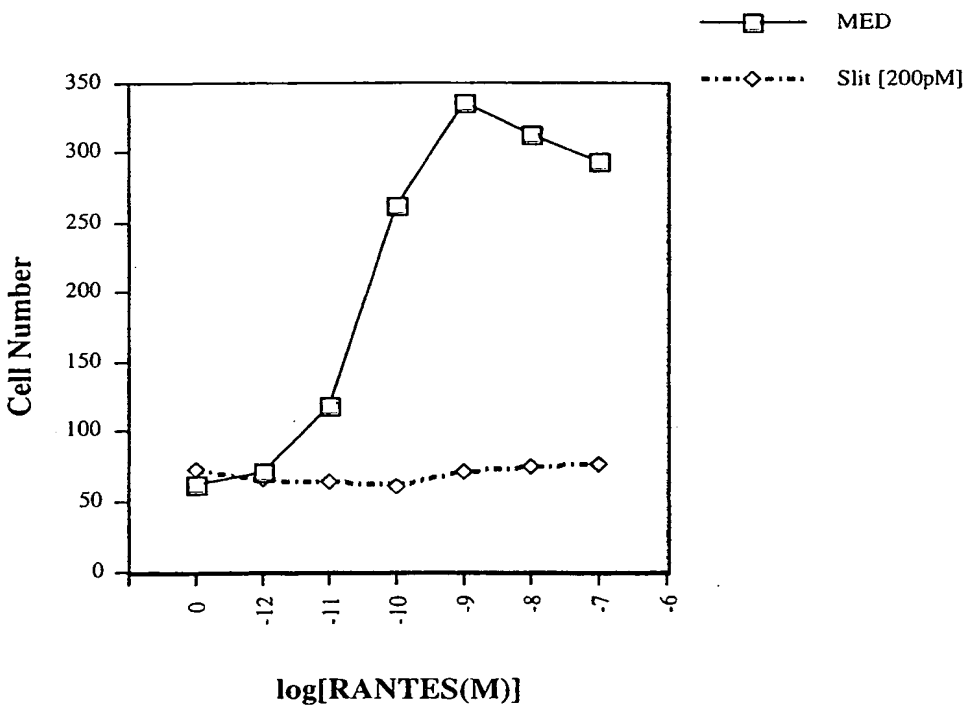
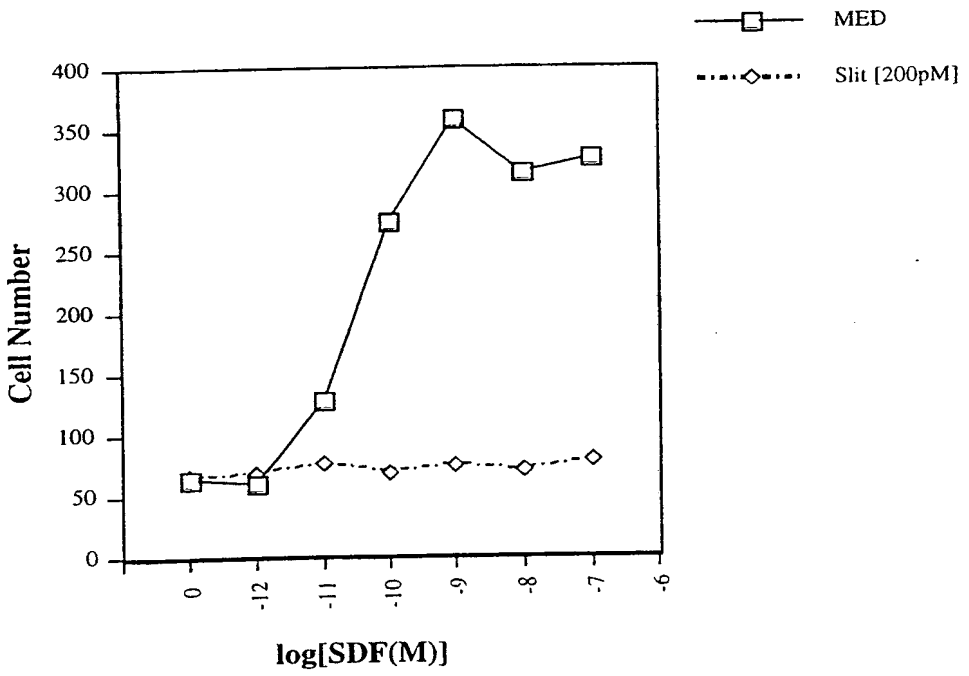

MED
Slit [200pM]
Cell Number
350
300
250
200
150
100
50
0
0
-12
-11
-10
-9
-8
-7
-6
log[RANTES(M)]

Fig. 13


MED
Slit [200pM]
Cell Number
400
350
300
250
200
150
100
50
0
0
-12
-11
-10
-9
-8
-7
-6
log[SDF(M)]

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60/124,767 17 March 1999 (17.03.1999) US

(71) Applicant: WASHINGTON UNIVERSITY [US/US]; One Brookings Drive, St. Louis, MO 63130 (US).

(72) Inventors: RAO, Yi; 12316 Ballas Pond Drive, Des Peres, MO 63131 (US). WU, Jane, Y.; 12316 Ballas Pond Drive, Des Peres, MO 63131 (US).

(74) Agents: BUTLER, James, E. et al.; Senniger, Powers, Leavitt & Roedel, 16th Floor, One Metropolitan Square, St. Louis, MO 63102 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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28 June 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: VERTEBRATE PROTEIN SLIT, DNA SEQUENCE ENCODING IT AND USES THEREOF

(57) Abstract: Polynucleotides that encode the Xenopus slit protein along with the deduced amino acid sequence of the Xenopus slit protein are given. Recombinant polynucleotides, vectors and transformed cells containing the slit polynucleotide are disclosed. Methods for production of slit proteins, pharmaceutical compounds containing slit protein and therapeutic uses for slit protein are also given. Methods for the repulsive guidance of nerve axon growth and inhibition of cell migration using slit are provided. Methods of stimulating cell proliferation using slit are given, along with methods and compositions for reducing the use of serum in cell culture by the use of slit.

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/475 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
STRAND, EPO-Internal, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GREG P. HOLMES ET AL.: "Distinct but overlapping expression patterns of two vertebrate slit homologs implies functional roles in CNS development and organogenesis" MECHANICS OF DEVELOPMENT, vol. 79, 1998, pages 57-72, XP000912208 cited in the application the whole document --- -/--	1-58

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2000	22 12 2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	MONTERO LOPEZ B.

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AKIRA ITOH ET AL.: "Cloning and expression of three mammalian homologues of Drosophila slit suggest possible roles for Slit in the formation and maintenance of the nervous system" MOLECULAR BRAIN RESEARCH, vol. 62, 1998, pages 175-186, XP000937694 cited in the application the whole document ---	1-58
A	TADAYOSHI HAYATA ET AL.: "Molecular cloning of XNLRR-1, a Xenopus homolog of mouse neuronal Leucine-rich repeat protein expressed in the developing Xenopus nervous system" GENE, vol. 221, no. 1, October 1998 (1998-10), pages 159-166, XP004143153 AMSTERDAM NL the whole document ---	1-58
A	WO 92 10518 A (YALE UNIVERSITY) 25 June 1992 (1992-06-25) page 5, paragraph 1 - paragraph 2 page 9, paragraph 3 -page 10, paragraph 1 page 44, paragraph 5 -page 47, paragraph 1 page 59, paragraph 2 -page 66, paragraph 3 ---	1-58
P,X	HUA-SHUN LI ET AL.: "Vertebrate Slit, a secreted ligand for the transmembrane protein Roundabout, is a repellent for olfactory bulb axons" CELL, vol. 96, no. 6, 19 March 1999 (1999-03-19), pages 807-818, XP002146822 NA US page 808, right-hand column, paragraph 2 -page 809, left-hand column, paragraph 1 page 810, left-hand column, paragraph 1 -page 811, left-hand column, paragraph 1 page 813, right-hand column, paragraph 1 -page 814, left-hand column, paragraph 1 page 815, left-hand column, paragraph 3 - paragraph 2 --- -/--	1-18,20, 21,28, 29,31, 32,40, 47,49

1

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 00/07040

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	J.-H. CHEN ET AL.: "Embryonic expression and extracellular secretion of Xenopus slit" NEUROSCIENCE, vol. 96, no. 1, 19 January 2000 (2000-01-19), pages 231-236, XP000900918 abstract page 231, right-hand column, paragraph 2 page 233, left-hand column, last paragraph -page 235, right-hand column, paragraph 1 -----	1-21,28, 29,31, 32,47,49

1

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/07040

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 16-40, and 50-58, as far as concerning an in-vivo method, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see additional sheet ,Invention 1

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-15, 17, 29, 32, 35, 36, 40, 42, 44, 47-49, 51, 54, 56, 58 and partially 16, 18-28, 30, 31, 33, 34, 37-39, 41, 43, 45, 46, 50, 52, 53, 55, 57

   Slit polynucleotide comprising SEQ ID NO:1 and variants thereof, vector, expression cassette and host cell comprising the same and use for producing the encoded polypeptide; encoded protein comprising SEQ ID NO:2, fragments and variants thereof; composition and cell culture medium comprising such protein; use of the polypeptide for altering cell migration, treating graft rejection, increasing cell proliferation, culturing cells, inhibiting HIV infection, aiding wound repair and organ regeneration and inhibiting inflammation

2. Claims: Partially 16, 18-28, 30, 31, 33

   Use of slit proteins other than as specified under subject 1 for altering cell migration

3. Claims: Partially 34, 37-39

   Use of slit proteins other than as specified under subject 1 for treating graft rejection

4. Claims: Partially 41, 43, 45, 46

   Use of slit proteins other than as specified under subject 1 for culturing cells and culture medium comprising it

5. Claims: Partially 50, 52

   Use of slit proteins other than as specified under subject 1 for inhibiting HIV infection of a cell

6. Claim : Partially 53

   Use of slit proteins other than as specified under subject 1 for aiding wound repair

7. Claim : Partially 55

   Use of slit proteins other than as specified under subject 1 for aiding organ regeneration

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

8. Claim : Partially 57

Use of slit proteins other than as specified under subject 1 for for inhibiting inflammation

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 00/07040

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9210518 A	25-06-1992	NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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(74) Agents: BUTLER, James, E. et al.; Senniger, Powers, Leavitt & Roedel, 16th Floor, One Metropolitan Square, St. Louis, MO 63102 (US).

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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Date of publication of the amended claims: 19 July 2001

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WO 00/55321 A3

AMENDED CLAIMS

[received by the International Bureau on 20 February 2001 (20.02.01);
original claims 10, 11 and 15 amended; remaining claims unchanged (2 pages)]

6. A host cell comprising the recombinant vector of claim 3.

7. The host cell of claim 6, wherein said host cell is selected from the group consisting of mammalian cells, plant cells, insect cells, yeast, bacteria, bacteriophage.

8. The host cell of claim 6, wherein said host cell expresses a protein encoded by said vector.

9. The host cell of claim 8, wherein said expressed protein is secreted by said host cell.

10. A protein or polypeptide fragment encoded by the polynucleotide selected from the group consisting of:

(a) a polynucleotide of SEQ ID NO: 1;

(b) a polynucleotide that has at least 90% sequence identity with the polynucleotide of (a);

(c) a polynucleotide that hybridizes to the polynucleotide of (a) under conditions of 5X SSC, 50% formamide and 42°C, and which encodes a protein having the same biological function;

(d) a polynucleotide encoding the same amino acid sequence as (a), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code;

(e) a polynucleotide encoding the same amino acid sequence as (b), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code; and

(f) a polynucleotide encoding the same amino acid sequence as (c), but which exhibits regular degeneracy in accordance with the degeneracy of the genetic code.

11. A protein comprising the amino acid sequence of SEQ ID NO: 2 or a fragment of SEQ ID NO: 2 and having the same biological function as the protein of SEQ ID NO: 2.

12. The protein of claim 10, wherein one or more of the amino acids have been substituted with a conserved amino acid and the biological function of the protein has been maintained.

13. The protein of claim 10, wherein one of more of the amino acid resides includes a substituent group.

14. A method for the production of a purified slit protein comprising growing host cells of claim 8 under conditions where said host cells express a protein encoded by said recombinant vector and isolating said expressed protein.

15. A pharmaceutical composition comprising the protein of claim 10, a protein fragment thereof having the same biological function, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier, diluent, or excipient.

16. A method for altering cell migration comprising administering a migration altering amount of a slit protein or a pharmaceutically acceptable salt thereof.

17. The method of claim 16 wherein said slit protein is the protein of claim 10.

18. The method of claim 16, wherein said alteration is by repulsion.

19. The method of claim 16, wherein said alteration is by inhibition.

20. The method of claim 16, wherein said cells express the roundabout (robo) protein.

21. The method of claim 16, wherein said cells are neural cells.

22. The method of claim 16, wherein said cells are malignant cells.

23. The method of claim 16, wherein said cells are leukocytes.